"MULTIVARIATE ANALYSIS OF TUBERCULOUS PLEURAL EFFUSION AND EVALUATION OF SEROLOGICAL RESPONSE TO A60 ANTIGEN COMPLEX"

Dissertation submitted to

Coimbatore Medical College for

M.D. Degree in Microbiology

Branch IV

THE TAMILNADU

DR. M.G.R. MEDICAL UNIVERSITY

CHENNAI

September 2006

CERTIFICATE

This is to certify that this dissertation work entitled **"Multivariate analysis of tuberculous pleural effusion and evaluation of serological response to A 60 antigen complex "** is a bonafide record of work done by **Dr. G. KALAISELVI,** in the DEPARTMENT OF MICROBIOLOGY, COIMBATORE MEDICAL COLLEGE AND HOSPITAL, COIMBATORE – 641 004, under the effective guidance of **Dr. R.K. GEETHA**, M.D.D.C.P., during the period of study (2003 – 2006).

DEAN **GUIDE**

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DECLARATION

I solemnly declare that the dissertation titled **"Multivariate analysis of tuberculous pleural effusion and evaluation of serological response to A 60 antigen complex ""** was done by me at Coimbatore Medical College Hospital during the period from October 2004 – September 2005 under the guidance and supervision of **Prof. Dr. R.K. Geetha,** M.D., D.C.P.

This dissertation is submitted to the Tamilnadu Dr. M.G.R. Medical University towards the partial fulfillment of the requirement for the award of M.D. Degree (Branch – IV) in Microbiology.

Place :

Date : **Dr.G. KALAISELVI**

ACKNOWLEDGEMENT

I am extremely thankful to **Dr. T.P. Kalaniti, M.D.,** The Dean, Coimbatore Medical College and Hospital for allowing me to use the hospital facilities for doing this study.

I express my deep sense of gratitude and heartfelt thanks to **Prof. Dr. R.K. Geetha, M.D., D.C.P.,** Professor and HOD of Microbiology for the whole hearted help given to me.

I take this opportunity to express my sincere thanks and indebtedness to **Prof. Dr.Anbu N. Aravazhi, M.D.,** for his constant encouragement, timely help in getting all the materials and continuous support throughout the course of this work.

I am ever grateful to **Prof. Dr. K. Rajendran, M.D.,** for his intellectual guidance and valuable suggestions rendered to me.

I hereby record my heartfelt and sincere thanks to **Dr.C.N. Paramasivam,** Deputy Director of Bacteriology Department, Tuberculosis Research Centre, ICMR, Chennai for his technical and skillful guidance offered to me to complete this project.

I express my sincere gratitude to **Dr. B. Sekar, M.D.,** Deputy Director Microbiology, Central Leprosy Teaching and Research Institute, Chengelpet for his voluntary help rendered to me in doing PCR work.

I wish to extend my special thanks to **Dr. M. Bhaskar, M.D.,** Assistant Professor, Department of Microbiology, Coimbatore Medical College, for guiding me through all my problems with constant encouragement and advice.

My gratitude and thanks to **Mrs. Selvanayaki and Mr. Padmanaban,** Laboratory Technicians for their magnanimous help in doing all investigations.

My sincere thanks to **Mrs. Nalini Sundaram,** Technical Officer, Bacteriology Department, TRC, ICMR, Chennai for her excellent job in bringing out all the tests.

Last but not the least I would like to thank my colleagues and all others in the department for their kind co-operation.

The huddles I have crossed in my life are due to the blessings of God and my parents, so I thank the almighty and my parents.

The immense help and co-operation rendered by my husband and my only charming daughter throughout my carrier remembered at this juncture.

Dedicated to my loving daughter.

CONTENTS

ABBREVIATIONS

M. tuberculosis	:	Mycobacterium tuberculosis
NTM	:	Non. tuberculous mycobacteria
MDR TB	:	Multi drug resistant tuberculosis
PT	:	Pulmonary tuberculosis
EPTB	:	Extra pulmonary tuberculosis
M. bovis	:	Mycobacterium bovis
AFB	:	Acid Fast bacilli
ZN Staining	:	Ziehl Neelsen staining
TPE	:	Tuberculous pleural effusion
HPLC	:	High performance liquid chromatography
PCR	:	Polymerase chain reaction
ATT	:	Anti tuberculous treatment
RNTCP	:	Revised national tuberculosis control programme
ELISA	:	Enzyme linked immuno sorbant assay

Introduction

INTRODUCTION

"As a destroyer of mankind, tuberculosis has no equal…."

Tuberculosis, “the captain of all the men of death”, a reemergent killer, is threatening to assume serious proportions all over the world. India is one of the worst affected countries bears about 28.4% of the entire world’s population. There are 15 million estimated cases in India with 8.8 million new cases, of which 3.9 million cases are smear positive. Every year 2.2 million contact tuberculosis and every second an Indian over 20 years of age is infected. (RNTCP statistical report, 2005).

Tuberculosis became a problem with the emergence of HIV/AIDS pandemic, because HIV not only reactivates a latent infection but also makes the disease more serious and treatment ineffective. Another factor that has made the situation more grave is the emergence of multidrug resistant tuberculosis (MDR TB). So serious is the global threat of tuberculosis WHO in 1993 took the unprecedented step of declaring this disease, a global emergency[6].

Extra Pulmonary tuberculosis is increasing worldwide. The global increase is believed to be fuelled by the HIV related immune competence,

more than 70% of the HIV positive individual with tuberculosis have had extra pulmonary presentation, while its prevalence is 15 – 30% in immuno compromised individuals[46].

Tuberculosis of the pleura is considered to be one among the severe form of extra pulmonary tuberculosis. It can occur alone or in combination with the pulmonary variety[112].

Pleural effusion caused by M. tuberculosis occurs in approximately 30% of patients with tuberculosis[46]. One of the common sequelae of pleural effusion is residual pleural thickening. Fibrothorax, Empyema thoracis, Empyema necessitans, pneumothorax, and pyopneumothorax are other complications[25,120].

The non-availability of technically simple, reliable, rapid and reproducible test often makes the diagnosis difficult. The traditional method of Mycobacterial culture, though considered as a gold standard is less sensitive and takes several weeks to become positive. In paucibacillary situations like tuberculous pleurisy even the sensitivity of smear examination is very low.

In circumstances where smear and culture are negative but when the clinical suspicion is high and corroborative evidences such as radiological and biochemical analysis of pleural fluid are apparent , other reliable tests with increased sensitivity may be needed. In the scenario of global resurgence of tuberculosis a rapid, sensitive and cost effective diagnostic test would aid in early diagnosis and prompt initiation of appropriate treatment there by limiting the spread of infection especially in the developing countries where vast majority of cases occur.

PCR is the rapid and sensitive method when compared to the traditional methods. It can amplify even a single bacilli present paucibacillary TPE. But the expensive laboratory set up and the non-availability of PCR at peripheral centers are the demerits of PCR as a routine diagnostic test.

Serological studies provide information concerning events of humoral immunity following Mycobacterial infection and also offer more pragmatic perspectives i.e., the possible early and rapid diagnosis of tuberculosis. ELISA techniques using various Mycobacterial antigens have been employed for the serodiagnosis of tuberculosis. A60 antigen complex appeared to be a dominant antigen during human infections. Several of its characteristics such as heat stability and immunodominance

make it as a perfect antigen candidate to be used in a diagnostic system[23]. The present study was undertaken to explore the usefulness of this novel antigen, A 60 antigen complex in the diagnostically difficult group of extra pulmonary tuberculosis, the tuberculous pleural effusion.

Aims and Objectives

AIMS AND OBJECTIVES

1. Isolation of Mycobacterium tuberculosis in Tuberculous pleural effusion employing conventional bacteriological methods.

2. Identification of Mycobacteria employing standard phenotypic methods.

3. Species level confirmation employing high performance liquid chromatography.

4. Identification of Mycobacteria employing molecular methods such as PCR.

5. Performing drug susceptibility on the isolates confirmed as Mycobacterium tuberculosis.

6. Screening of the study subjects for the presence of HIV infection.

7. To evaluate the diagnostic potential of the A60 antigen complex by ELISA in Tuberculous pleural effusion patients.

Review of Literature

REVIEW OF LITERATURE

3.1.TUBERCULOSIS

Definition:

Tuberculosis is a chronic necrotizing bacterial infection with a wide variety of manifestations caused by Mycobacterium tuberculosis complex. Although, tuberculosis can affect any organ of the body, the lung is virtually always the portal of entry[120].

History:

Tuberculosis must have been a scourge since mankind existed; as reports of this dreadful disease are recorded in the vedas and it was called 'rajayakshma' (meaning wasting disease). Hippocrates called the disease "pthisis', a Greek word which meant, "to consume" (460 – 377 B.C.). Evidence of tuberculosis lesions of bone has also been found in Egyptian mummies (3400 B.C.).

J.L. Schonlein, is credited to have name the disease tuberculosis (Latin Word: Tubercula) which means "a small lump". Oliver Wendell Holmes referred to the disease as "white plague". Tuberculosis has been ever present and is resurging with a Vengeance.

Robert Koch first identified the tubercle bacilli in 1882. He succeeded in cultivating the bacillus on inspissated serum and transmitted the disease to many animals of different species by inoculation with pure cultures of the bacillus. This became the basis of "Koch's Postulates".

Mycobacteria are not readily stained by the Gram method as the high lipid content of the cell wall. Once stained, they are not easily decolourized, this resistance to decolourization is called "acid fastness". In 1882, Robert Koch stained the tubercle bacilli with hot methylene blue. Ehrlich discovered the "acid fast" property of the bacteria in 1882 and stained the bacilli with hot fuchsin in the presence of aniline oil as a mordant. In 1883 Ziehl changed the mordant to phenol and Neelsen in 1884 combined the dye and mordant to form carbol fuchsin. This acid fast staining technique is known as the "Ziehl – Neelsen Method".

Cold Staining technique (kinyoun) as well as the use of fluorescent dyes to visualize the Mycobacteria have also been developed. In 1885 Nocard isolated the avian form of the tubercle bacilli. In 1889 Smith described the bovine variety.

Etiological agent:

Mycobacterium is currently the only genus in the family Mycobacteriaceae, order Actinomycetales[130]. The minimum standards for including a species in this genus are :

- Acid – alcohol fastness.
- The presence of Mycolic acids containing 60 – 90 carbon atoms.
- G + C content of the DNA of 61 – 71 mol% (Levy - Frebault and protaels 1992).

Mycobacteria are straight or slightly cured rods about 0.2 - 0.6 X 1.0 – 10 μm (Wayne and kubica 1986) non-motile, non-sporing, aerobic or microaerophilic. Colony morphology varies among the species, M. tuberculosis forms 'eugonic' growth with rough, tough and buff colonies while M. bovis is 'dysgonic' with smooth, moist, white colonies which break up easily when touched. NTM forms colonies which are non pigmented (non Photochromogens) to pigmented (Yellow to orange, rarely pink) some species require light to form pigment (Photochromogens) while others produce pigment either in the light or in the dark (Scotochromogens) (Timpe and Runyon 1954). Nutritional requirement and temperature range vary considerably.

M. Tuberculosis Complex:

Tuberculosis of man and animals is caused by a group of very closely related species forming M. Tuberculosis complex[130]. They are

- M. tuberculosis – the human tubercle bacillus
- M. bovis – the bovine tubercle bacillus
- M. microti – the vole tubercle bacillus
- M. africanum – strains of human origin occurring in Africa
- M. canettii – a new taxon, variant of M. tuberculosis characterized by smooth colonies (Van Scolingen 1997 Pfyffer 1998, Mittgen 2002 et al;)

Transmission:

The tubercle bacilli are commonly discharged as aerosols of pulmonary secretion and are transmitted in air born droplet nuclei (1 to 10 μm)[58]. One bout of cough produces 3,000 droplet nuclei and these can stay in the air for a long period of time. The development of disease depends upon the size of the inoculum, duration of exposure, infectivity of the Mycobacterial strain and immune status of the host.

Epidemiology:

Global forecast of tuberculosis during 1990 – 2000 shows 10.2 million new cases of tuberculosis occurred world wide in addition to the 8.22 million existing cases which includes 42% in the South East Asia, 24% in the Western Pacific region, 14% in the Sub Saharan Africa, 9% in the Europe, 7% in the American region and 4% in the Eastern Mediterranean region, 75% of tuberculosis cases in developing countries are in the productive age group (15-50 Yrs)[98]. The disease infects 1% of the world's population each year.

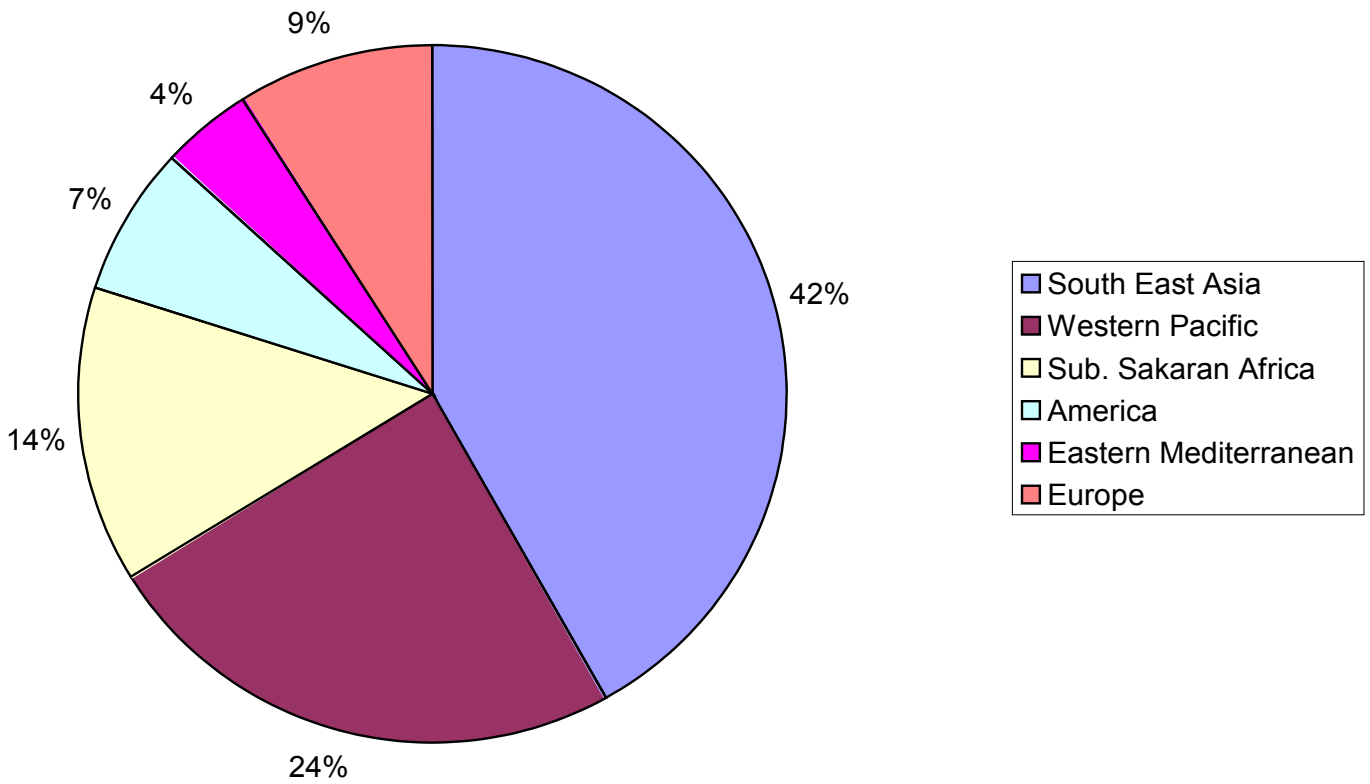
Epidemiological Indices:

About 40% of the Indian population are infected with TB. Incidence of tuberculosis is about 1.5 per 1,000 populations per year. It increases with age and is more common in males. The prevalence of bacteriologically confirmed disease was 4 cases per 1,000 populations.

3.2. NON TUBERCULOUS MYCOBACTERIA:

Mycobacteria, other than human or bovine tubercle bacilli which may occasionally cause human infection, have been called non tuberculous Mycobacteria (NTM). NTM infections become more prevalent in relation to the AIDS pandemic(Brown – Elliott et al; 2002).

Global Forecast of tuberculosis by WHO report 1997



It can cause pulmonary infections in the immuno compromised hosts (Rigsby and Curtis 1994). NTM rarely cause Tuberculous pleural effusion but cases have been reported caused by M. kansasii[73], M. gordonae[122] and M. intracellulare[48].

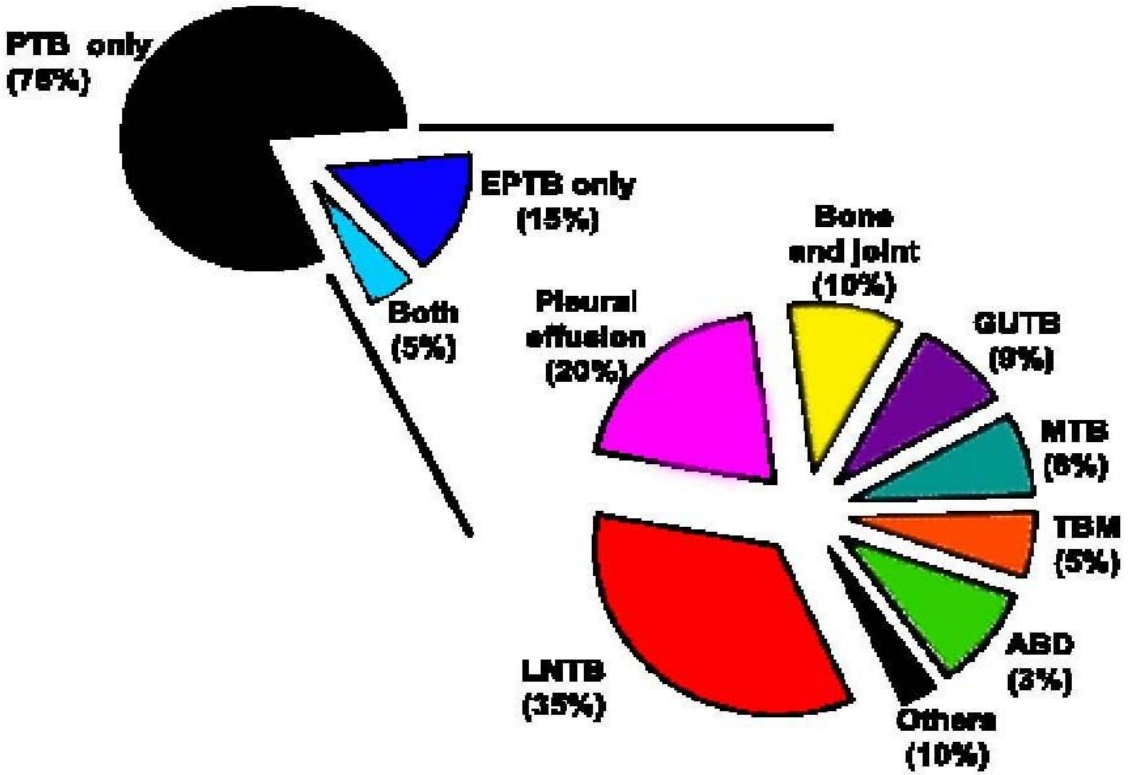
3.3.EXTRA PULMONARY TUBERCULOSIS:

Although virtually all organs may get affected, the order of frequency of involvement are the Lymph nodes, Pleura, Bone & Joints, Genitourinary tract, Miliary, Meninges and peritoneum[118].

TUBERCULOUS PLEURAL EFFUSION:

Definition:

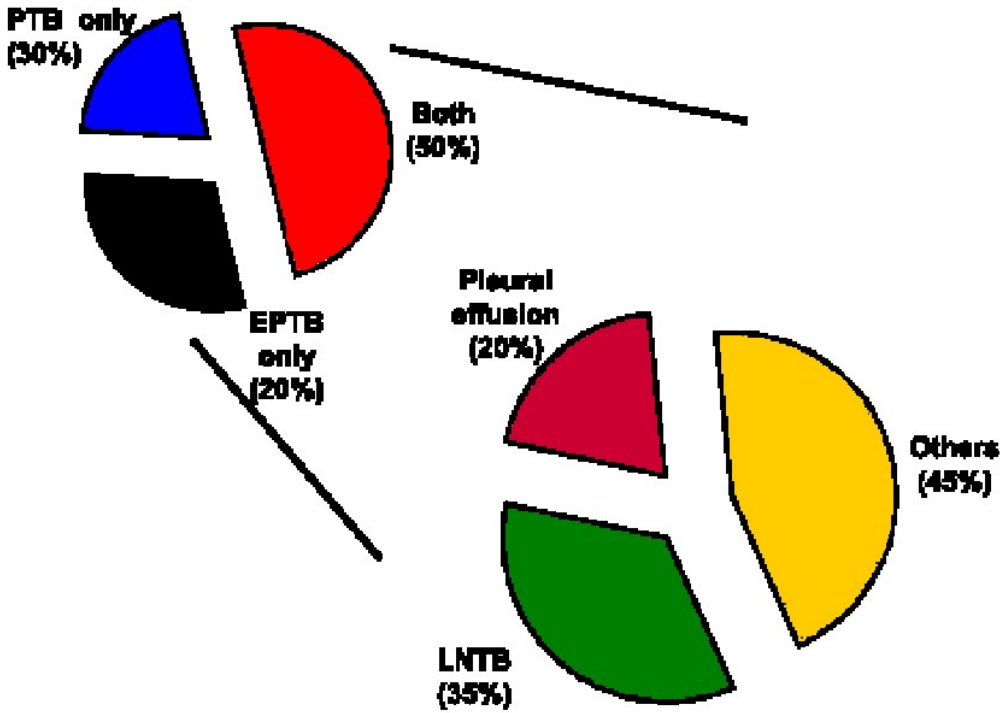
Accumulation of significant amount of fluid in the pleural cavity is called "Pleural Effusion". Normally this means serous effusion (Exudate). No pleural effusion can be detected in most of the healthy individual although about 15ml may be found in 10% of the population. Atleast 300 – 500 ml of fluid must be present in the pleural cavity to be detected clinically as well as radiologically.



Distribution of tuberculosis cases by anatomical site in HIV – negative patients.

PTB - Pulmonary tuberculosis, EPTB – Extra pulmonary tuberculosis, GUTB – Genitourinary tuberculosis, MTB – Miliary tuberculosis, TBM – Tuberculosis meningitis, ABD – Abdominal tuberculosis, LNTB - Lymph node tuberculosis.

(Source : S.K. Sharma & A. Mohan et. al., 2004)



Distribution of tuberculosis cases by anatomical site in HIV – positive patients.

PTB - Pulmonary tuberculosis, EPTB – Extra pulmonary tuberculosis, LNTB - Lymph node tuberculosis.

(Source : S.K. Sharma & A. Mohan et. al., 2004)

Pathophysiology:

Normally protein free fluid flows from the systemic capillaries in the parietal pleura to the pleural space and then to pulmonary capillaries in the visceral pleura (amount of fluid is upto 500ml/hr)[3].

Protein Exchange:

Normally pleural fluid contains approximately 1.5g/100ml and this protein increases with inflammation. If protein accumulates, the colloid osmotic pressure of the pleural fluid increases and the pressure gradient favours the movement of fluid from both pleural layers into the pleural space[76].

Pathogenesis:

Classically Tuberculous pleural effusion was described in children and young adults who do not have an apparent pulmonary lesion. However as many as 50% now occurs in conjunction with pulmonary tuberculosis[120]. The pleural infection is always secondary to tuberculosis elsewhere, normally in the lung or mediastinal lymph node, reaching the pleura by direct extension (rupture of sub pleural focus), by lymphatic spread or by haematogenous dissemination[125]. Because the tubercle bacilli are found to be very few in effusions, the severe pleural reaction

may be due to hypersensitivity to the pleural tissues, which develop after primary tuberculosis infection[81,9].

Diagnosis of Tuberculous Pleural Effusion:

Diagnosis of TPE is usually based on clinical signs and symptoms and radiography. In addition demonstration of Mycobacteria in pleural fluid remains the gold standard method.

Clinical Diagnosis:

TPE most often manifests as an acute illness with complaints of chest pain (Pleuritic in nature), non productive cough and dyspnoea. Other constitutional symptoms includes fever (Low grade), loss of appetite, loss of weight, haemoptysis and clubbing[15].

Clinical signs includes fullness and diminished movement of the chest, stony dull note on percussion, markedly diminished or absent breath sounds. Mediastinal shift & tracheal shift to the opposite side noted in massive effusion.

Radiological Diagnosis:

The chest radiography usually demonstrates a unilateral pleural effusion and is usually small to moderate in size. Bilateral pleural effusion occurs in <10% of cases and indicates Miliary Tuberculosis. A

parenchymal lesion may be seen in 30 – 50% of cases[1,110,45]. Ultra sonography[12] and C.T. scan[61] are useful in the diagnosis of encysted effusion (In India, tuberculosis is the most common cause of encysted effusion).

Laboratory Diagnosis:

This can be broadly divided into

(1) Demonstration and isolation of Mycobacterium tuberculosis.

(2) Identification and typing of M. tuberculosis.

(3) Demonstration of host's response to exposure to M. tuberculosis.

Sputum Smear Examination:

Yield of sputum examination in a patient with pleural effusion is quite high if underlying lung parenchymal lesion is present. Even in the absence of underlying lung infiltrates sputum examination may be positive in 4 – 11% of patients with tuberculous pleural effusion[13,30,109].

Composition of Pleural Fluid[78]:

	Normal	Tuberculosis
Volume	< 15ml	>300 – 500 ml
Cell/mm^3	100 – 500	500 – 2,500
Mesothelial Cells	3 – 70%	Absent
Lymphocytes	30 – 75%	>90%
Granulocytes	10%	Early stages
Glucose	Plasma level	> 60mg/dl
LDH	< 50% plasma level	> 200 IU
PH	≥ Plasma	> 7.3
Protein	1 – 2 g/dl	> 3 g/dl
ADA	----	70 IU/L.

Protein pleural fluid/serum ratio > 0.5

LDH Pleural fluid / serum ratio > 0.6[80,81]

STAINING METHODS:

(1) Ziehl – Neelsen Staining (ZN)

Microscopy provides a simple, sensitive and rapid means of detecting M. tuberculosis but in paucibacillary conditions ZN stain is positive only if number of AFB is more than 10,000/ml of specimen.

SPECIAL STAINING FOR AFB:

Fluorescent Microscopy[113]

Auramine dye is a fluorochrome, which can be raised to a higher energy level after absorbing ultraviolet light. When the dye molecules return to their normal lower energy state they release excess energy in the form of visible (fluorescent) light. Auramine requires blue excitations light, exciter filters that select for light in the 450 – 490 λ wavelength range and a barrier filter for 515λ. Brightly fluorescent bacilli will appear yellow against a dark background[17]. Its sensitivity and specificity are fairly similar to ZN microscopy[113].

Mycobacterial Culture:

For detection of Mycobacteria in clinical specimens the current "gold standard" consists of a combination of solid and liquid media. The specimen after processing may be inoculated into Egg based solid medium (Lowenstein – Jensen Medium) and Middle brook 7H9 broth medium. In detecting as few as 10^1 - 10^2 viable organisms/ml of specimen, culture is more sensitive than smear. About 4 – 8 weeks may be required before the growth is detected. M. Tuberculosis produces eugonic buff, rough and tough colonies.

DRUG SUSCEPTIBILITY TESTS:

Three general methods[26,62]:

- The Absolute concentration Method: It involves standard inoculum on media containing graded concentration of drugs and resistance is expressed in terms of MIC – minimum inhibitory concentration.

- The Resistance Ratio Method: Compares the growth of known strain and test strain in various dilutions of drug media and resistance is expressed in terms of ratio of test strain / standard strain.

- Proportion Method: This method enables precise estimation of the proportion of the bacilli resistant to a given drug. If > 1% of strains show resistance compared to control at a critical concentration, the strain is considered as resistant.

- The proportion of bacilli resistant to a given drug is then determined by expressing the resistant portion as a percentage of the total population tested.

3.4.IDENTIFICATION AND TYPING METHODS OF MYCOBACTERIA:

Typing Methods of Mycobacterium Tuberculosis:

The traditional methods of identification of Mycobacteria based on morphological and biochemical properties. The current identification scheme mainly relies on molecular methods along with phenotypic tests.

Phenotyping of M. Tuberculosis:

Phenotypic tests include determination of preferential growth, temperature, pigmentation, photoreactivity and colony morphology.

Tests	M. Tuberculosis	Others
Growth Rate	Slow	Slow / Rapid
Temp. Requirement	35°c – 37°c	25°c – 37°c
Pigmentation	Absent	Present / absent
Niacin	Positive	Mostly negative
Stability of catalase at 68°c	Negative	Positive
Growth PNB Media	Negative	Positive

Identification of NTM:

Non-Tuberculous Mycobacteria are usually preliminarily identified by traits such as growth rate and pigmentation according to Runyon's criteria (Runyon 1959).

Runyon's Classification[106]:

Runyon Group	Growth Rate	Pigmentation	Typical Members
I	Slow (> = 5 Days)	Photochromogenic (Yellow or Orange pigment after exposure to light)	M. kansasii M. marinum
II	Slow (> = 5 Days)	Scotochromogenic (Yellow or Orange pigment in the dark)	M. scrofulaceum.
III	Slow (> = 5 Days)	Non chromogenic (no pigment in the dark or in the light)	M. avium M. intra cellulare
IV	Rapid (< = 4 days)	Variable	M. fortuitum complex M. smegematis M. phelei

Biochemical Tests:

The most useful biochemical tests for the precise characterization of species with in Mycobacterium tuberculosis complex are the niacin production, thermostable catalase at 68°c, aryl sulphatase, nitrate reductase and phosphatase activity.

Mycolic acid analysis (HPLC):

Mycolic acids are present in all Mycobacterium. Their composition is constant for all strains of a given species and varies from species to

species (Minnikin et al; 1980; Daff'e et al; 1983). A High performance liquid chromatography method (HPLC) for analysis of Mycolic acid esters has been standardized and demonstrated to be a rapid and reliable method for identification of many Mycobacterial species.[14,22,52,128,129].

MOLECULAR TYPING OF M. TUBERCULOSIS:

PCR strategy targeting IS 6110.

Detection of Mycobacterial DNA by PCR has the advantage of increased sensitivity and specificity. It has been suggested that those systems that amplify DNA sequences present in multiple copies are more sensitive than those that amplify targets present in a single copy. PCR using the IS6110 repetitive sequence as a probe is considered the gold standard for typing M. tuberculosis. IS 6110 is a 355 – bp repetitive insertion sequence that displays variability both in the copy number (from 0 to 25) and chromosomal position[130]. This repetitive target increases the sensitivity of assay allowing detection of smaller number of bacilli.

Serodiagnosis of M. Tuberculosis:

M. Tuberculosis is an intra cellular pathogen that evokes both cellular and humoral immune response. Eventhough it is the former that

determines the outcome of an infection, it is the later which is mainly used in diagnostic purposes.

Cellular Immune response and delayed hypersensitivity reaction:

CMI can be defined as a beneficial host response characterized by an expanded population of specific T Lymphocytes that in the presence of microbial antigens produces cytokines locally. These cytokines attract macrophages from the blood stream and activate them. Tumour Necrosis Factor - α and interferon γ are major macrophage activating cytokines[21]. Interferon γ (IFN – γ) also induces interleukin – 2 receptors in macrophages[107]. Activated macrophage produce reactive oxygen & nitrogen intermediates, lysosomal enzymes and other factors that kill and digest tubercle bacilli.

HUMORAL IMMUNE RESPONSE:

The cell wall of Mycobacterium:

Mycobacteria have a complex outer envelope composed of several distinct layers (Brennan and Draper 1994). Inner most layer is the plasma membrane inserted into it are proteins, mannosides and lipoarabinomannan.

Next is the peptidoglycan (murein) layer formed by complex network of polysaccharide chains cross-linked by tetrapeptide chains containing aminoacids. A prominent feature of the outer surface of Mycobacteria is its high lipid content which accounts for upto 60% of the cell wall weight, composed of glycolipids associated with the long chain fatty acids termed Mycolic acid. Proteins are found throughout the various layers.

A60 Antigen Complex:

A60 antigen complex is a thermo stable macromolecular antigen (TMA) found in the cytosol of Mycobacteria. This complex is composed of roughly $1/3$ proteins, $1/3$ carbohydrates and $1/3$ lipids, exact proportions being dependent on culture stages[23,50]. Researchers also determined that the peptide fraction of A60 contains approximately 30 distinct bands in the range 20 – 80 kDa as separated by the SDS page method[47]. The A60 antigen complex is composed of about 3 proteinic epitopes of which 8 were identified by monoclonal antibodies (Lipoarabinomannan, a glycolipid and proteins of 65,40,38,35,19 and 14 kDa).

Species Distribution of A60 Antigen:

A60 antigen complex extracted from M. bovis is genus specific. A60 from M. bovis and M. tuberculosis were 98% homologous.

Humoral Immune Response to A60 antigen complex:

The humoral response elicited by the A60 antigen complex are directed against its protein components (Galot and Wene 1994). During human disease about 90% of the antibody detected were directed against Antigen 60. A60 thus appeared to be a dominant antigen during human diseases. In addition it has been demonstrated that 2 microgram of A60 induces the same cellular reaction as 5 units of PPD.

3.5.MANAGEMENT OF TUBERCULOUS PLEURAL EFFUSION:

According to Revised National Tuberculosis Control Program (RNTCP), short course chemotherapy based on two months of 3 or 4 drugs followed by 2 drugs for the next 4 months have been found to be highly effective in patients with Tuberculous pleural effusion. The WHO is now implementing the Directly Observed Treatment Short Course (DOTS) in places with a high incidence of tuberculosis[88,97]. By ensuring cure, this strategy aims at reducing the number of infectious cases in the community and also preventing the emergence of drug resistance. Fluoroquinolones particularly ofloxacin has good penetration into the pleural fluid and hence can be used in the management of patients with

drug resistant Tuberculous effusion[145]. Steroids can be used as a adjunct to ATT in severely ill patients[7].

Materials and Methods

MATERIALS AND METHODS

This study was carried out at Department of Microbiology, Coimbatore Medical College, Coimbatore, Tamil Nadu from October 2004 to September 2005.

4.1. STUDY POPULATION:

All the Patients who were clinically diagnosed as pleural effusion got admitted during the study period with pleural effusion in the Department of Medicine, Coimbatore Medical College Hospital, Coimbatore were considered for the study. Those patients who fulfilled the following inclusion criteria were included as study population

Inclusion criteria:

1. Radiological evidence of pleural effusion with or without pulmonary infiltrates (Plate I).
2. Ultrasound and C.T scan (Thorax) evidence of pleural effusion.
3. Biochemical analysis of pleural fluid demonstrated straw colured fluid, total count more than 500 cells /mm^3, lymphocytic predominance (> 80%) and pleural fluid glucose more than 60 mg/ dl.

105 patients who fulfilled the above criteria were included in the study. Among the 105 cases 10 were < 20 years and 95 were >20 years of age. 71 were male and 34 were female.

4.2. SAMPLE COLLECTION:

Pleural Fluid:

Under strict aseptic precautions, about 20ml of pleural fluid was collected and distributed into four sterile anticoagulated screw capped bottles (Plate II).

I First sample was utilized for biochemical analysis and looked for glucose, protein and chloride.

II Second sample was utilized for cytology and cell count such as total count and differential count.

III Third sample was utilized for bacteriological examination: Grams stain, AFB stain, bacterial Culture and Mycobacterial culture were done.

IV Fourth sample was stored at -20ºc for PCR.

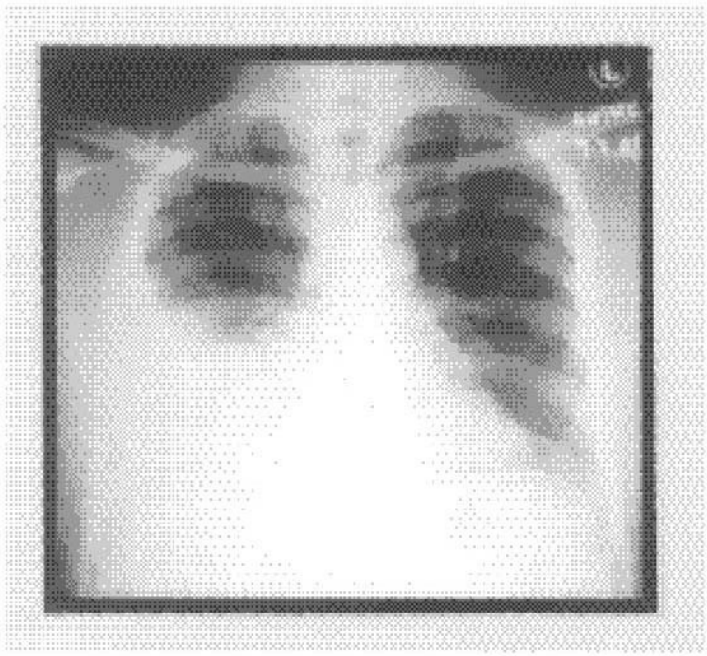


Plate I

Chest Radiograph (Postero anterior view) showing right sided pleural effusion

(Source : S.K. Sharma, 2004)

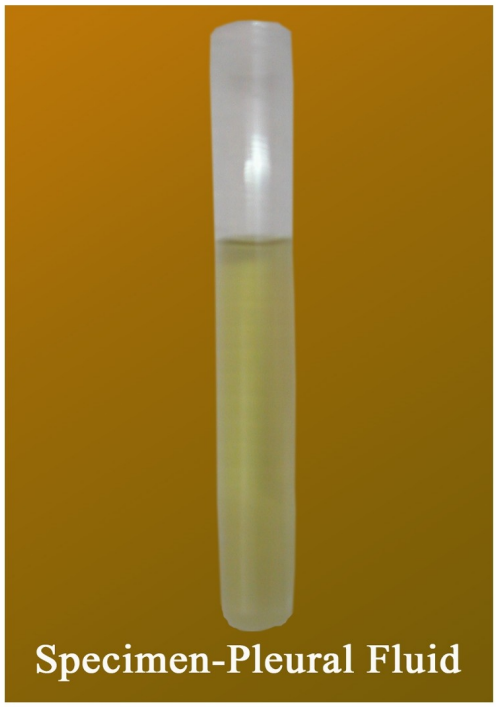


Plate II

Pleural Fluid

Sputum Sample:

Three consecutive early morning spontaneously induced sputum samples were collected in sterile screw capped bottles.

Serum Sample:

Under aseptic precautions 5ml of blood sample was collected by venepuncture, serum separated, aliquoted and stored -20°c till the time of use.

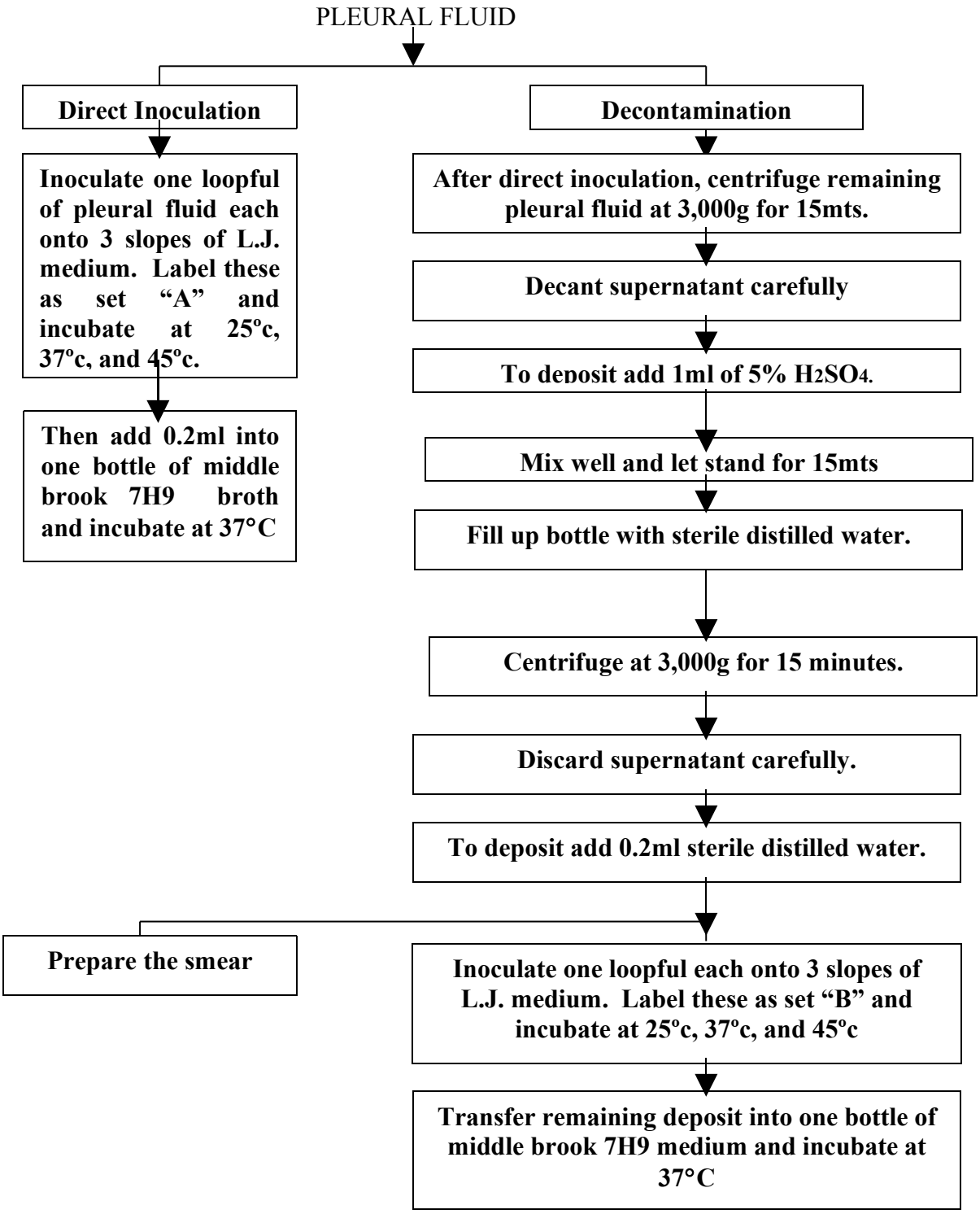
4.3. LABORATORY SAFETY[139]:

All the safety measures were taken during sample collection. Sterile, leak proof, unbreakable and properly labeled containers were used. A microbiological safety cabinet (class 1) open fronted with exhaust was used. 1% Sodium hypochlorite was used as disinfectant solution. Cleaning of the laboratory was done with clear phenolic disinfectants and the room was periodically fumigated with formaldehyde.

4.4. PREPARATION OF SPUTUM SMEAR:

Sputum smear preparation and examination were done in the same way as described under microscopic examination of pleural fluid.

SAMPLE PROCESSING:



4.5. PLEURAL FLUID ANALYSIS:

Macroscopic Examination:

All the 105 pleural fluid samples collected from clinically diagnosed cases were macroscopically examined for colour and turbidity (clear or serosanguinous). Cytological examination and biochemical analysis were also done in all the samples.

MICROSCOPIC EXAMINATION:

Preparation of Smears[125]:

The slides used for preparation of smears were clean, grease free and pre sterilized. The specimen number was marked on the slide using a diamond pencil. A separate new slide was used for each specimen. By using a 5 mm internal diameter 24 SWG Nichrone wire loop, the centrifuged deposit of the specimen was smeared as a uniform layer over the slide. Finally the slides were air dried and then heat fixed in the safety cabinet.

Modified Ziehl Neelsen Staining Procedure[84] :

1. Placed the slide on a staining rack with the smeared side facing up.
2. Flooded entire slide with strong carbol – Fuchsin.
3. Heated the slide slowly until it was steaming for 5mts by using intermittent heating.

4. The slide was rinsed in a gentle steam of running water until all free stain was washed away.
5. Flooded the slide with acid alcohol mixture, as a decolorizing agent for 2 to 3mts.
6. Rinsed the slide thoroughly with water. Drained excess water from the slide.
7. Flooded the slide with methylene blue counter stain for 30 sec.
8. Rinsed the slide thoroughly with water, allowed the smear to air dry.

Recording and Reporting of Results :

For Ziehl – Neelsen's stained smears, the following semi quantitative method of reporting is recommended.

No. Of Acid fast bacilli (AFB)	Fields	Report
No AFB	In 100 immersion fields	Negative
1 – 9 AFB	In 100 immersion fields	Record exact figure
10 – 99 AFB	In 100 immersion fields	1 +
1 – 10 AFB	Per field (examine 50 fields)	2 +
> 10 AFB	Per field (examine 50 fields)	3 +

4.6. CULTURE METHODS [4,86]:

Preparation of Lowenstein Jensen Medium

Mineral salt solution with malachite green:

The properly weighed mineral salts were dissolved in about 75ml of distilled water by heating. Added 3ml of glycerol to it and then made up to 150ml with distilled water. Sterilization was done by autoclaving at 121˚c for 30 minutes.

Homogenized whole eggs:

Fresh hen's eggs not more than 7 days old were cleaned by scrubbing thoroughly with hand brush using soap and water . The eggs were soaked in soap solution for 30 minutes , rinsed thoroughly in running water and then again soaked in 70% ethanol for 15mts. After washing the hands thoroughly the eggs were cracked at the edge of the beaker and poured in to a sterile flask and beaten with a sterile blender.Homogenized egg solution was prepared by filtering the egg solution into a measuring jar using sterile funnel and gauze.

Preparation of Complete Medium:

About 150ml of mineral salt solution with malachite green was mixed thoroughly with 250ml of homogenized egg solution. The media

was then distributed in 6 to 8 ml quantities in sterile universal containers and the caps were closed tightly.

Coagulation of Medium:

The bottles were placed in a slanted position in the inspissator (85˚c) for 1 hour to coagulate the medium. They were stored at room temperature for over night and again reinspissated for 30 minutes at 85˚c on the next date. Sterility of the media was checked by incubating at 37˚c for 24 hours (Plate III).

Preparation of Middlebrook 7H9 broth Medium:

The properly weighed salts were dissolved in 450ml of distilled water by heating, if necessary and 2ml of glycerol was added to it. Sterilization was done by autoclaving at 15 lbs pressure at 121˚c for 10 minutes. After cooling to 45˚c, one vial (50ml) of Middlebrook ADC growth supplement was added aseptically and mixed well. The media was then distributed in 5ml amounts in sterile screw capped bottles. Sterility was checked by over night incubation at 37˚c (Plate IV).

Plate III
Lowenstein Jenen Medium

Plate IV

Middle Brooke 7H9 Medium

4.7.INOCULATION:

A loopful of centrifuged sediments of the specimen was inoculated over the middle surface of the medium by using a 5mm loop.

4.8. INCUBATION OF CULTURE:

All cultures were incubated at 25°c, 37°c and 45°c.

4.9. CULTURE EXAMINATION:

The cultures were examined weekly to detect any rough, buff and tough (cauliflower like growth) colonies. If growth was present, it was reported and send for further identification. If no growth, incubation was continued till 8 weeks and reported as negative.

4.10. CULTURE REPORTS:

Culture reports recorded were qualitative (Positive or negative) as well as quantitative (number of colonies isolated).

Reading	Report
No growth	Negative
1 - 19 colonies	Positive (Number of colonies)
20 - 100 colonies	Positive (1+)
> 100 discrete colonies	Positive (2+)
Confluent growth	Positive (3+)
Contaminated	Contaminated

4.11. DRUG SUSCEPTIBILITY TEST[26]

The absolute concentration Method:

A standardized inoculum grown on drug free media and media containing graded concentration of the drugs were tested. Several concentration of each drug was tested and resistance was expressed in terms of the lowest concentration of the drug that inhibits growth i.e., minimum inhibitory concentration.

Drug free LJ medium:

Drug free LJ fluid medium was prepared as mentioned before. The graded concentration of various drugs were added as follows and then inspissated to coagulate the medium (Plate V).

STREPTOMYCIN - S.

Stock solution:

250 mg of streptomycin sulphate in 20ml of distilled water. (10,000 µg/ml)

Working solution:

4 ml of 10,000µg/ml + 16ml water = 2,000 µg/ml.

1 ml of 2,000µg/ml + 19ml water = 100 µg/ml.

Media preparation:

ML of stock		LJ Medium (ML)	Final Concentration (µg/ml)
µg	ml		
2.0	100	100	2
4.0	100	100	4
2.4	2000	600	8
4.8	2000	600	16
9.6	2000	600	32
3.84	10000	600	64

ISONIAZID: (INH)

Stock solution:

200mg Isoniazid in 20ml distilled water (10,000 µg/ml). Sterilized by membrane filtration.

Working Solution:

ML of Stock (µg/ml)	Water (ml)	µg/ml
1 ml of 10,000	9	1,000
2 ml of 1,000	18	100
1 ml of 100	19	5
2 ml of 5	8	1

Media preparation:

Stock (µg/ml)	LJ medium (ml)	µg/ml
2.5 ml of 1 µg/ml	100	0.025
5ml of 1 µg/ml	100	0.05
2ml of 5 µg/ml	100	0.1
1.2ml of 100 µg/ml	600	0.2
6ml of 100 µg/ml	600	1.0
3ml of 1,000 µg/ml	600	5.0

RIFAMPICIN (R)

Stock solution:

200mg rifampicin in 20ml of dimethyl formamide (10,000 µg/ml).

Working solution:

4 ml of 10,000 µg/ml + 16ml water = 2,000 µg/ml

2 ml of 2,000 µg/ml + 18ml water = 200 µg/ml

Media preparation:

Stock (µg/ml)	LJ medium (ml)	µg/ml
2.0 ml of 200 µg/ml	100	4
4 ml of 200 µg/ml	100	8
8 ml of 200 µg/ml	100	16
9.6 ml of 2,000 µg/ml	600	32
3.84 ml of 10,000 µg/ml	600	64
7.68 ml of 10,000 µg/ml	600	128

ETHAMBUTOL (E)

Stock solution:

270mg Ethambutol in 20ml of distilled water (10,000 µg/ml). Sterilized by membrane filtration.

Working solution:

Stock (µg/ml)	Water (ml)	µg/ml
2 ml of 10,000	18	1,000
10 ml of 1,000	10	500
1 ml of 5,00	9	50

Media preparation:

Stock (µg/ml)	LJ medium (ml)	µg/ml
1.0 ml of 500 µg/ml	100	0.5
2 ml of 500 µg/ml	100	1.0
2.4 ml of 500 µg/ml	600	2.0
4.8 ml of 500 µg/ml	600	4.0
4.8 ml of 1,000 µg/ml	600	8.0

Inoculation:

A bacterial suspension was prepared by adding approximately 4mg moist weight of a representative sample of the bacterial mass visualized, (as ⅔ loopful of 3mm internal diameter 24 SWG wire loop) into 0.2ml of sterile distilled water in 7ml Bijou bottle containing twelve 2 to 3 mm

glass beads. Shake the bottle for 1 minute to produce a uniform suspension, then add 0.8ml of sterile water and again shake the bottle. This suspension contains approximately 4 mg/ml of the organism.

Using a 3mm external diameter 27 SWG nichrome wire loop, inoculated one loopful of this suspension on each slope of the sensitivity test media. Setup three control drug free media (1 for control and 2 for catalase & niacin tests). Tested the standard sensitive strain H37 Rv with each batch of test. Incubated all slopes at 37˚c except one control drug free slope, which was incubated at 25˚c.

Reading:

The inoculated slopes were examined for growth after 28 days of incubation. Growth is defined as the presence of 20 or more colonies on a slope.

Interpretation of tests:

The lowest concentration of the drug that inhibits growth (MIC) was recorded. The ratio of the MIC of the test train to the MIC of the strain H37 Rv set up with each batch of tests is referred to as the resistance ratio (RR).

Definition of resistance

Drug	Level of resistance
Streptomycin	Resistance ratio of drug conc 8µg/ml or more
Isoniazid	MIC of drug conc 5 µg/ml or more
Rifampicin	MIC of drug conc 128 µg/ml or more
Ethambutol	MIC of drug conc 8 µg/ml or more

–

4.12. IDENTIFICATION TESTS:

1. Susceptibility to p. nitro benzoic acid (PNB).
2. Niacin Test.
3. Catalase activity at 68˚c /pH 7.

1. SUSCEPTIBILITY TO P. NITRO BENZOIC ACID

Stock solution

One gm of PNB was dissolved in 40ml of dimethyl formamide and mixed thoroughly which was added to entire quantity of 2liters of LJ fluid giving a final concentration of 500mg/l. The media was distributed and inspissated.

Procedure:

A loopful of bacterial suspension was inoculated on one slope of LJ medium without drug and 1 slope of LJ medium containing PNB at a conc. of 500 mg/l and incubated at 37˚c and the reading were taken after 28 days.

Interpretation:

M. Tuberculosis does not grow on PNB medium.

2. NIACIN TEST[138]

Reagents:

1. O. Toludine 1.5%
2. Cyanogens Bromide solution 10%.

Procedure:

The positive culture tube was taken and autoclaved at 120˚c for 30 minutes for the extraction of Niacin. 0.25ml of extract was taken and added to solution of 0.25ml of O. toludine and 0.25ml of 10% cyanogens bromide and mixed well in a screw capped test tube. Observed the solution for the formation of pink color (positive) within 5 minutes.

Interpretation

M. tuberculosis showed positive Niacin test (Plate VI).

Plate V

Drug susceptibility test

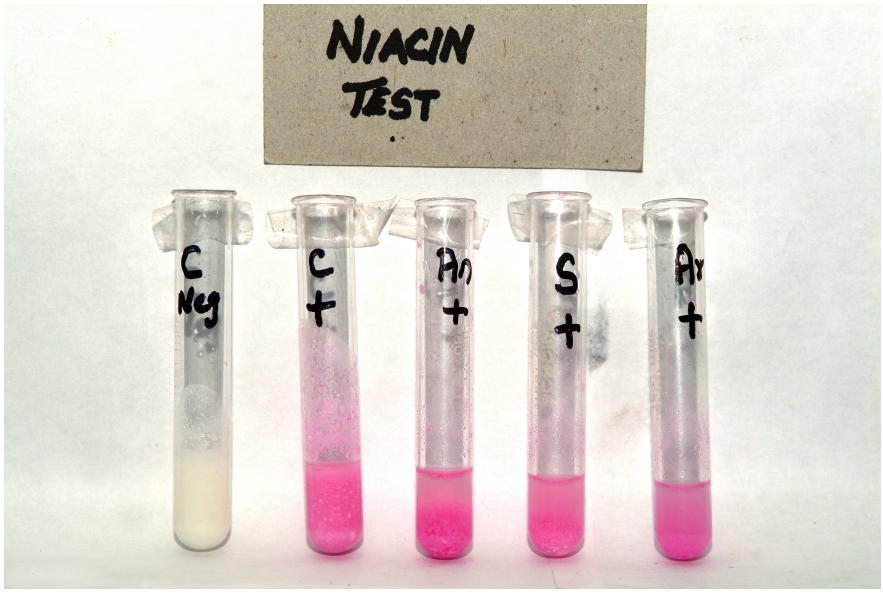


Plate VI

M. tuberculosis showing positive Niacin Test

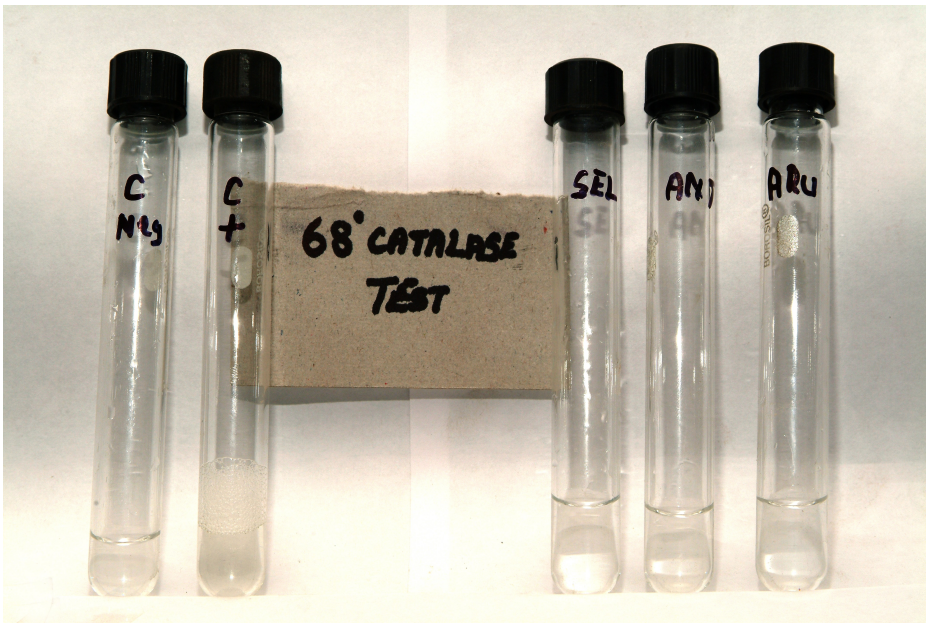


Plate VII

M. tuberculosis showing negative catalse at 68ºC

Plate VIII

High performance Liquid chromatography

3. CATALASE TEST[67]:

Reagent:

1. 0.067M phosphate buffer solution, pH7.0

   $Na_2H\ Po_4$ anhydrous 9.47g.

   Distilled water 1 litre.

   Dissolved Na2Hpo4 in water to provide 0.067M solution (solution 1)

   $KH_2\ Po_4$ 9.07g

   Distilled water 1 litre

   Dissolved KH2 Po4 in water to give 0.067M solution (solution 2)

   Mixed 61.1 ml of solution 1 with 38.9ml of solution 2.

2. Hydrogen peroxide 30%
3. Tween80 - 10% .

Procedure:

Added 0.5ml of 0.067M buffer to screw capped test tubes and emulsified with a loopful of test culture. Placed the tubes in a water bath at 68˚c for 20 minutes. After cooling to room temperature added 0.5 ml of freshly prepared equal parts of 10% tween80 and 30% H2O2. Observed the formation of bubbles appearing on the surface of the liquid. Keep negative tubes for 20 minutes.

Interpretation:

M. tuberculosis showed catalase negative at 68˚c (Plate VII).

4.13. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Principle:

The objective of this method is to identity Mycobacteria by analysis of Mycolic acids using HPLC. A suspension of AFB is saponified to cleave the Mycolic acids bound to cell wall. Mycolic acids are then separated by acidification and extraction into chloroform. After conversion to UV absorbing p. bromophenacyl esters the Mycolic acids are analyzed on a reverse phase C18 column using HPLC. A gradient of methanol and dichloromethane generated by micropressor controlled pumps is used to separate the Mycolic acid esters, which are detected with a UV spectrophotometer. Reproducible chromatographic pattern containing combination of different diagnostic peaks are formed. Pattern recognition is by visual comparison of sample results with Mycolic acid patterns from reference species of known Mycobacteria.

Specimen:

MTB grown on LJ medium, verified by ZN smear.

SAMPLE PREPARATION:

A. Cell harvesting Procedure

0.2ml of saponification reagent was added to 1 – 2 loopful of growth on LJ medium. Vortex for 20 sec.

B. Saponification and Extraction:

Autoclaved the mixture at 121˚c, 15 PSI for 1 hour, then added 0.2 ml of chloroform & 1.5 ml of acidification reagent (Conc. Hcl), Capped tightly and vortex for 20 sec. Allowed the layers to separate in 20 – 30 sec. Using a glass Pasteur pipette removed the bottom (chloroform) layer (containing Mycolic acids) and transferred to a new tube. Evaporated the chloroform in a water bath at 85 - 105˚c.

C. Derivitization to p. bromophenacyl esters:

Added 0.1 ml KOH & evaporated at 85 - 105˚c. The sample ws cooled and then added 1.0 ml of chloroform followed by 50 µl of Derivitization reagent. Capped & vortex for 30 sec. and again heated at 85 – 105˚c for 20 minutes.

D. Clarification by liquid. liquid extraction:

The sample was cooled and added 1.0 ml of clarification reagent. Vortex for 20 seconds. The bottom (chloroform) layer was removed and transferred to a new tube.

E. Completion and Storage:

Evaporated to dryness at 85 - 105˚c. Capped tightly and stored at 4 - 6˚c in the dark.

F. HPLC Analysis of Mycolic acid:

Mycolic acids were analyzed on a reverse – phase C18 column using HPLC. A gradient of methanol and dichloromethane (methylene chloride) generated by micro processor controlled pump was used to separate the Mycolic acid esters which were detected with a UV spectrophotometer (Plate VIII).

4.14. PCR FOR M. TUBERCULOSIS (TARGETING IS 6110)

Protocol:

The chromosomal DNA from pleural fluid was extracted by enzymatic methods. A 123bp fragment of the M. tuberculosis specific gene amplified by PCR with primers IS 6110. The most commonly used genetic maker is the Mycobacterial insertion element IS 6110. The target is repeated 8 -20 times with in the genome in M. tuberculosis complex. This repetitive target increases the sensitivity of assay allowing detection of smaller number of bacilli. PCR carried out in 50µl of a reaction mixture, 200 µM of dNTP's, IU Taq polymerase, 20 pmoles of each set of

primers and 10ng of chromosomal DNA. Samples are then subjected to initial denatnuration at 94ºc for 4 min followed by 35 cycles of 90ºc for 1 min, 72ºc for 1 min, and a final extension at 72ºc for 10 min to complete the elongation of the PCR intermediate products. PCR products are then run on 2% agarose gel containing ethidium bromide and examined for the presence of the 123bp and compared with 100bp ladder.

4.15. A60 BASED IMMUNO ASSAY:

ELISA Test procedure:

The ELISA plate with 96 micro wells coated with antigen 60 from M. bovis which has been provided was taken and the assay was arranged. Separate plates for IgG, IgM and IgA were provided along with controls. The references are ready to use were 2 and 16 u references for IgG, 4 U and 16U references for IgA, positive and negative control for IgM were added to A1 and B1 corresponding wells (100µl). 100µl of 1: 100 diluted serum samples were added to the subsequent wells in order. Incubated at 37˚c for 1 hour. After that the wells were washed with 1: 20-diluted wash buffer. Then added 100µl of POD - conjugate solution in each well including A1and B1. Incubated the plates at 37˚c for 30 minutes. Again washed the plates 5 times. Then added 100µl of 1:10 diluted tetramethylbenzidine (TMB) solution in each well including A1 and B1. Incubated at 37ºc for 15 minutes in the dark. Then added

Plate IX

ELISA test

Plate X

ELISA Kit

100 µl of stop solution. Read the ELISA plate absorbance at 450nm in ELISA Reader (Plate IX).

Cut off Values:

IgM : Positive > 1.0.

IgA : Positive > 350serounits.

IgG : Positive > 225serounits.

4.16. DETECTION OF HIV IN STUDY SUBJECTS:

Test procedure (Microlisa)

The ELISA plate with 96 micro wells coated with HIV recombinant proteins which has been provided was taken and the assay was arranged. A-1 served as the reagent blank. From well A-1 all the controls were arranged in a horizontal and vertical configuration. 100µl of the sample diluent was added to A-1 well as blank. Then added 100µl of negative control in each well numbered B-1 and C-1 respectively. Added 100µl of positive control in D-1, E-1 and F-1 wells. Then added 100µl of each sample diluted in sample diluent (sample 20µl and sample diluent 200µl as 1 : 11) in each well, starting from G-1 well. Then incubated at 37˚c for 30minutes. After 30 mins washed wells 5 times with working wash solution (25X). Then added 100 µl of working conjugate solution (1: 100 dilution) in each well including A-1. Incubated the plates at 37ºc for 30 mins. Washed the plates again 5 times. Then

added 100µl of working substrate solution (1 : 100 dilution) in each well including A-1. Incubated at room temperature (20˚c to 30˚c) for 30 mins in dark. Then added 50µl of stop solution. Read the ELISA plate absorbance at 450 nm with in 30 mins in ELISA reader after blanking A-1 well (Plate X). The odd value of 0.458 was obtained.

Results

RESULTS

The tests were performed as described in materials and methods. The results obtained are as follows.

Table 1

Age and Sex distribution of clinically diagnosed TPE (N = 105).

Age	Male	Female	Total
< 20yrs	6	4	10
20-50yrs	49	25	74
>50yrs	16	5	21

Of the 105 cases, 10 (10%) were <20yrs, 74(70%) were in between 20-50yrs and 21(20%) are above 50yrs. Maximum cases were recorded in the age group between 20 and 50yrs (Figure 1). In all the age groups the sex distribution was predominantly male(71%) with a ratio of 2:1 (Figure 2).

All the patients included in the study were belonging to low socio economic status. 20% of them were working in the textile industries and 10% of them were residing in an area close to textile units. Their educational status was poor. Co morbid conditions like smoking; alcohol, diabetes, hypertension and previous exposure to tuberculosis were found in 48 (46%) (Figure 3) of patients.

Table :2

Categorisation of Patients with predisposing factors (n= 48)

Criteria	No of Cases
Smoking	15
Alcohol	10
Previous Exposure to TB	3
Over –Crowding	7
Diabetes	5
Hypertension	5
Other Respiratory illness	3

5.1. DETAILS OF BACTERIOLOGICAL FINDINGS

Table 3

Sputum smear examination (N = 105).

Category	Number of cases	ZN Positive
Unilateral PT with effusion	40	5
Bilateral PT with effusion	10	10
Pleural effusion without pulmonary lesion	55	---

Figure - 1
Age distribution of clinically diagnosed TPE (N = 105)

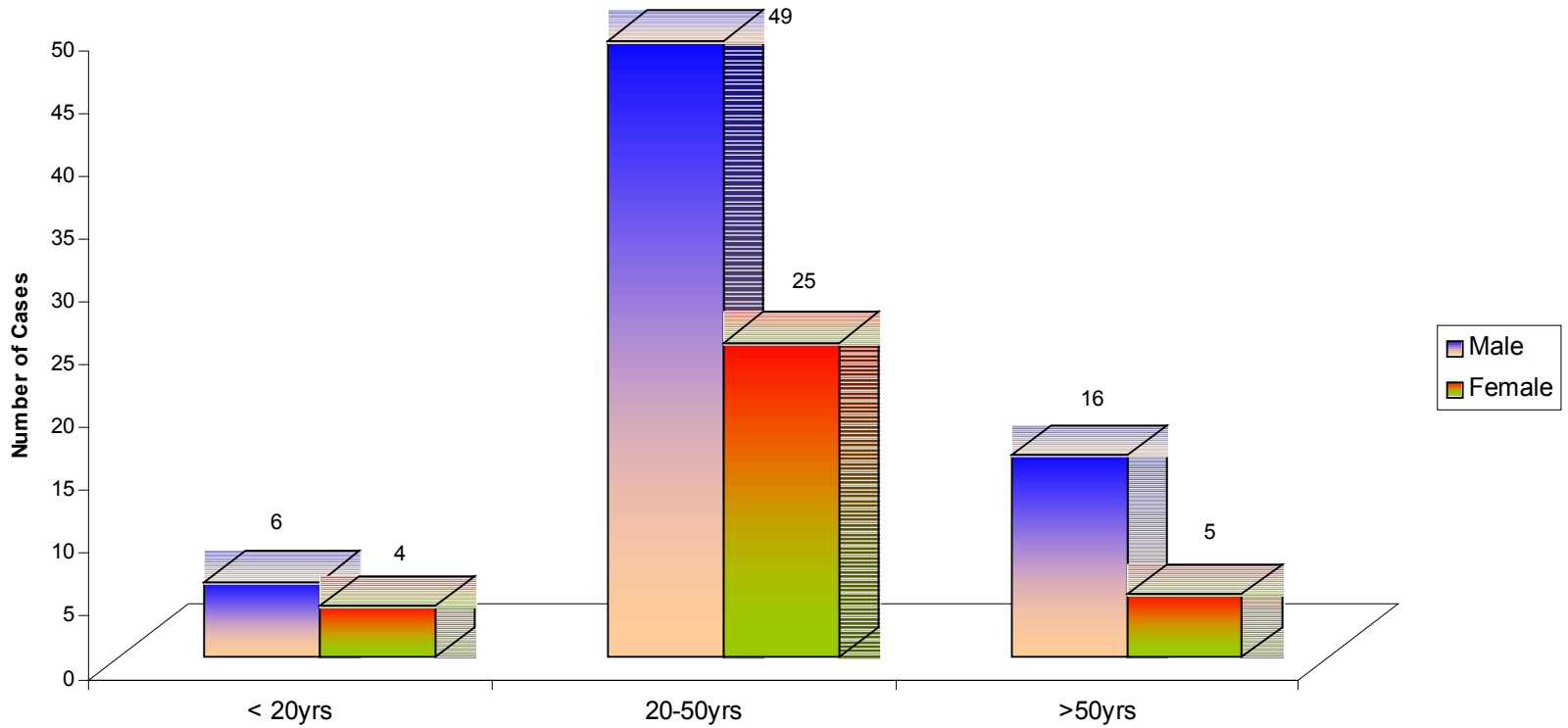


Figure - 2
Sex distribution of clinically diagnosed TPE (N = 105).

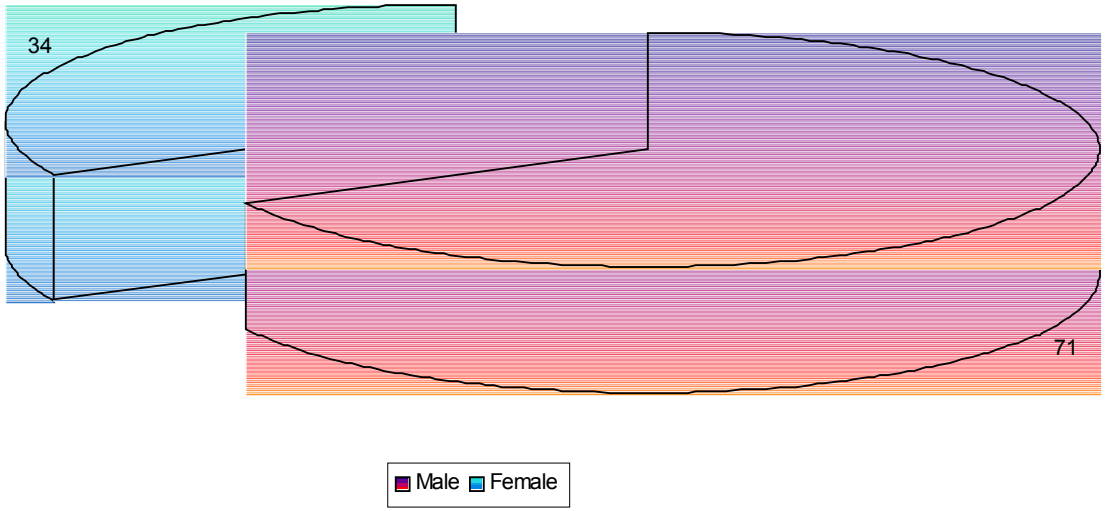


Figure - 3
Categorisation of Patients with predisposing factors (n= 48)

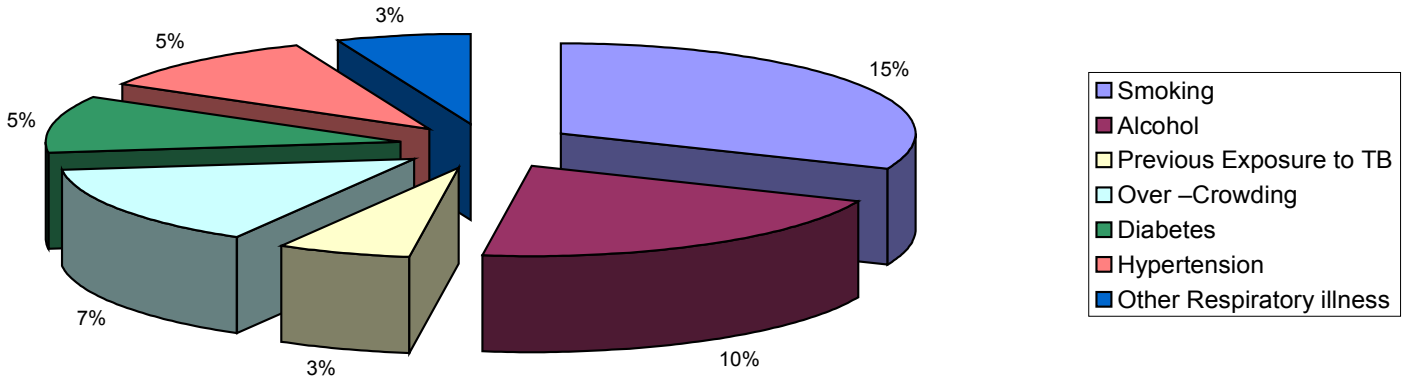
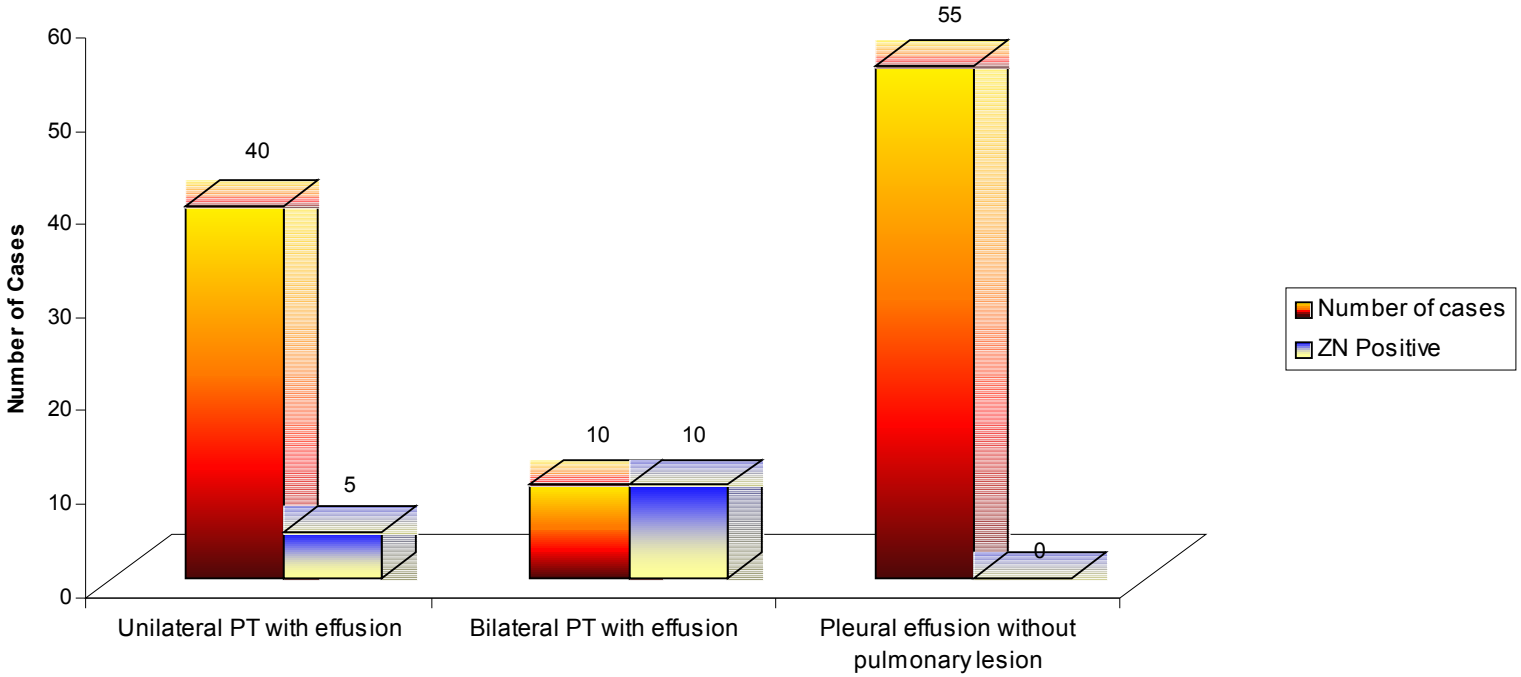


Figure - 4
Sputum smear examination (N = 105).



Among the 105 cases, 5 (4.8%) cases were ZN positive in patients with unilateral PT with effusion, 10(9.5%) cases were ZN positive in patients with bilateral PT with effusion and none of the cases from pleural effusion without pulmonary lesion (Figure 4).

5.2. BACTERIOLOGICAL PROFILE

Macroscopic Examination of the pleural fluid samples were clear and straw coloured but cloudy or serosanguinous fluids were also obtained in few cases. Pleura

l fluid volume ranges from 500ml to 2 liters. Cytological examination showed lymphocytic predominance (80 to 90%) in 85% of cases with total count varied

betwe
en
500 -
2000
cells /
mm^3.
Pleura
l fluid
protei
n
ranges
from
5 –
7gm/d
l and
glucos
e from
60 –
80
mg/dl.

Gram stain and bacterial cultures of the pleural fluid samples were negative.

Table 4

Pleural fluid smear examination (N = 105)

Category	Number of cases
Smear positive	4
Smear negative	101

Out of the 105 clinically diagnosed cases, 4 cases (3.8%) were smear positive (Plate XI) and the remaining 101 cases (96.2%) were smear negative (Figure 5).

Table 5

Pleural fluid culture examination (N = 105)

Category	Number of Cases
Culture Positive	21
Culture Negative	84

Among 105 clinically diagnosed TPE, 21(20%) cases were culture positive (Figure 6).

Table 6

Distribution of culture positive cases (N = 21)

Category	Number of cases
Mycobacterium tuberculosis	19
Non Tuberculous mycobacteria	2

Among the 21 culture positive cases, 19 cases (90%) were Mycobacterium tuberculosis and 2 cases (10%) were NTM (Scotochromogens) (Plate XIII, XIV).

Figure - 5
Pleural fluid smear examination (N = 105)

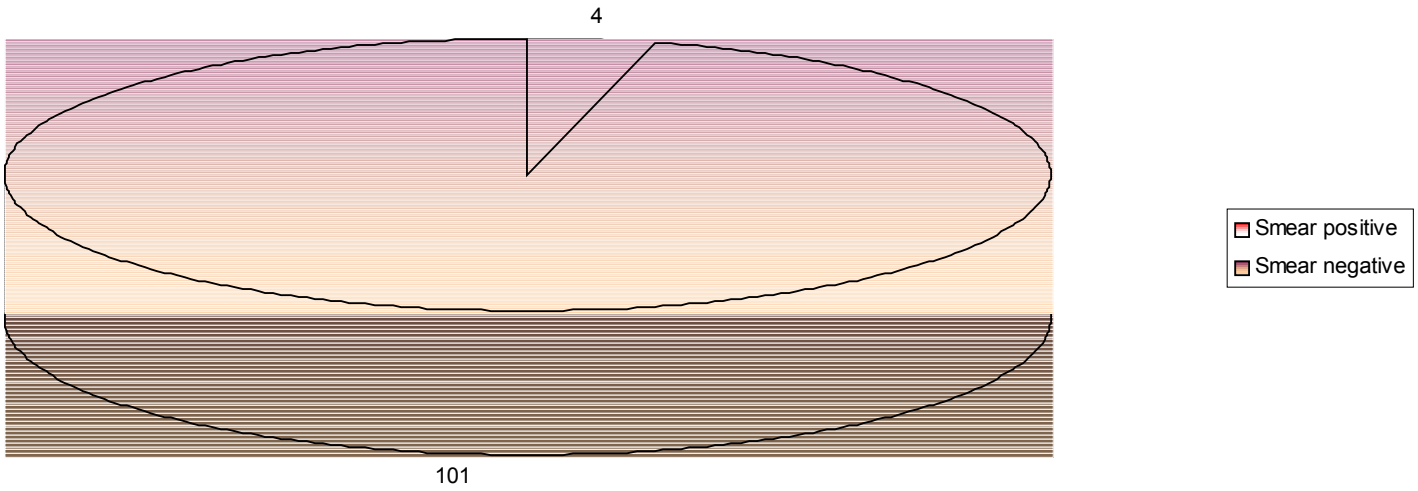


Figure - 6
Pleural fluid culture examination (N = 105)

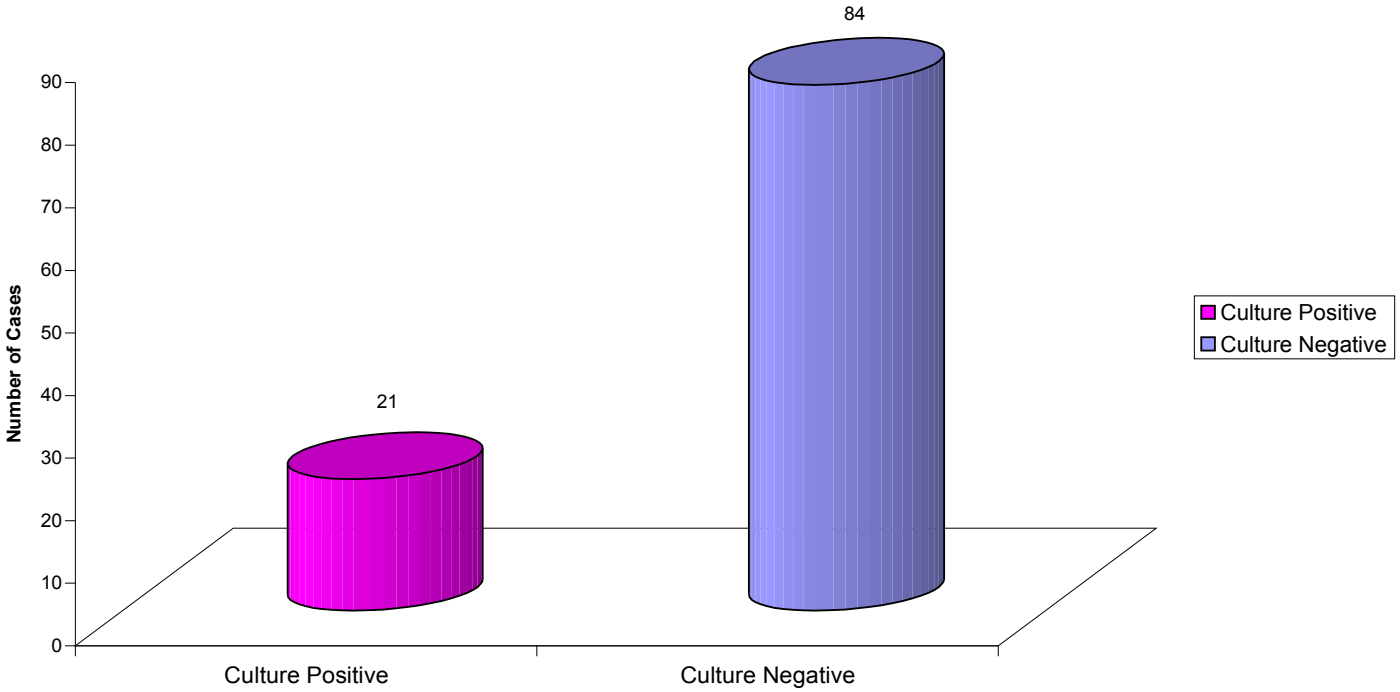


Table 7

Bacteriological profile of clinically diagnosed TPE (n = 105)

Category	Culture Positive	Smear positive	Smear negative
Mycobacterium tuberculosis (19)	19	3	16
Non Tuberculous mycobacteria (2)	2	0	2
Culture negative (84)	0	1	83

Among the 19 culture positive M. tuberculosis cases, 3 (2.8%) were smear positive (Plate XII). ZN was negative in 2 culture positive cases of NTM. Among the 84 culture negative cases 1 (1%) case was smear positive.

Table 8

The drug susceptibility pattern in the culture positive cases (N = 21).

Number of culture positive cases.	Drug conc. for standard sensitivity				PNB 500 µg/ml	Response to tests.
	Streptomycin (resistance ratio)	Isoniazid MIC	Rifampicin MIC	Ethambutol MIC		
17	< 2µg	<0.2µg	<32µg	<2µg	Sensitive	Sensitive to all drugs
4	<2µg	>5µg	<32µg	>8µg	Sensitive	Multi Drug Resistance

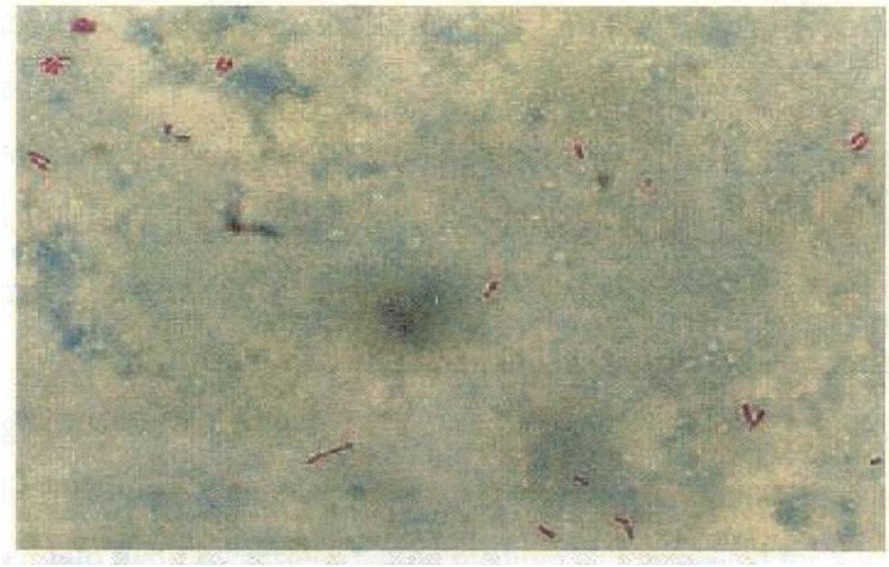


Plate XI

Pleural fluid smear showing Acid alcohol fast bacilli

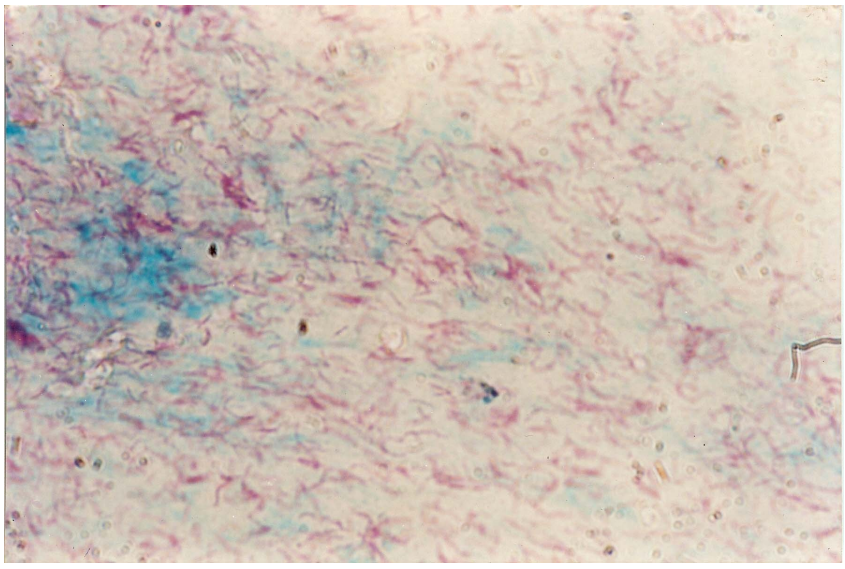


Plate XII

Pleural fluid growth showing acid fast bacilli

Plate XIII

L.J. Medium Showing growth of Mycobacterium tuberculosis

Plate XIV

L.J. Medium Showing growth of Non-tuberculous Mycobacterium

Out of the 21 culture positive cases, 17 cases were sensitive to all first line drugs indicated by no growth on the medium containing required and minimal concentration of drugs. Four cases were Multi drug resistant tuberculosis (Plate XV, XVI).

Species level confirmation of all positive cultures were done by HPLC (Plate XVII).

5.3. RESULTS OF PCR

Table 9

Category	S+C+	S+C-	S-C+	S-C-
Number of cases	3	1	16	85
PCR Positive	3	1	16	53

Among the 105 cases, PCR was positive in all culture positive 19 (18%) cases, PCR was positive in a single smear positive case (1%) and in the remaining culture and smear negative cases PCR was positive in 53 cases (51%) (Figure 7, Plate XVIII).

Figure - 7
Results of PCR

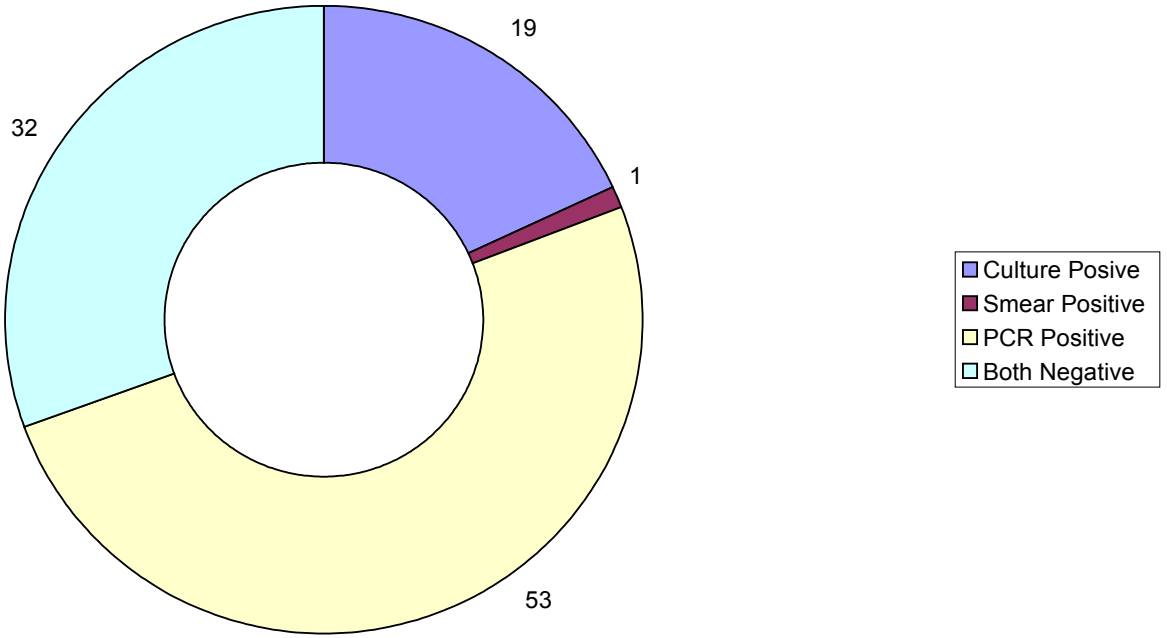


Figure - 8
Results of serology

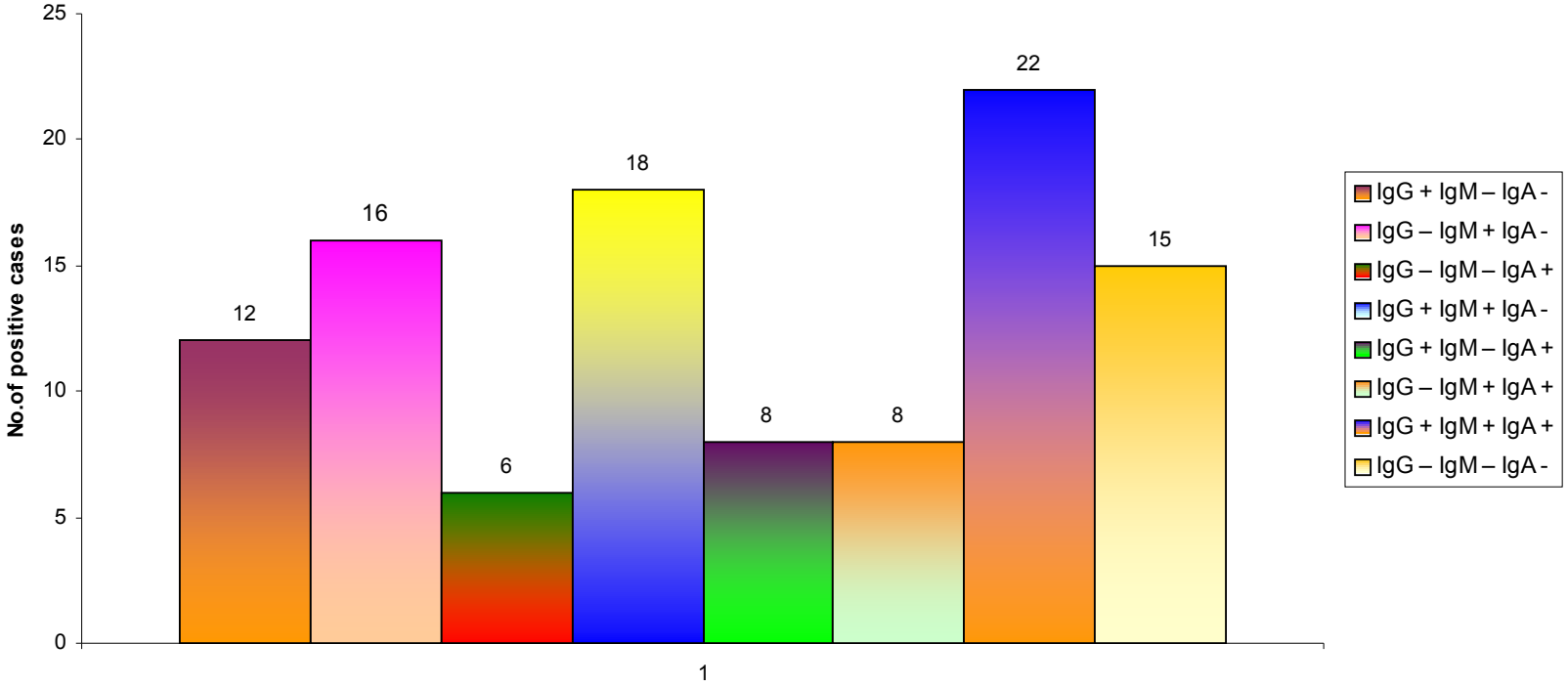


Plate XV

Drug susceptibility test showing drug resistance

Plate XVI

Drug susceptibility test showing drug resistance to Ethionamide

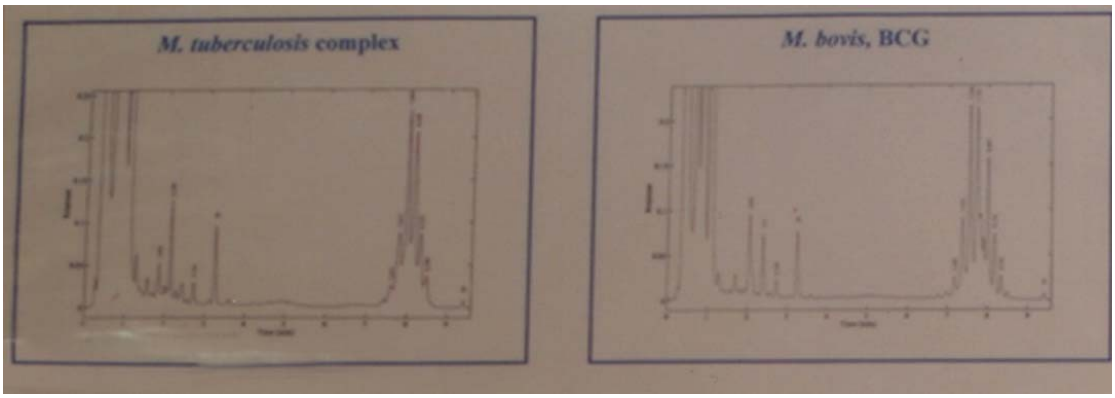


Plate XVII

Chromatography picture of M. tuberculosis

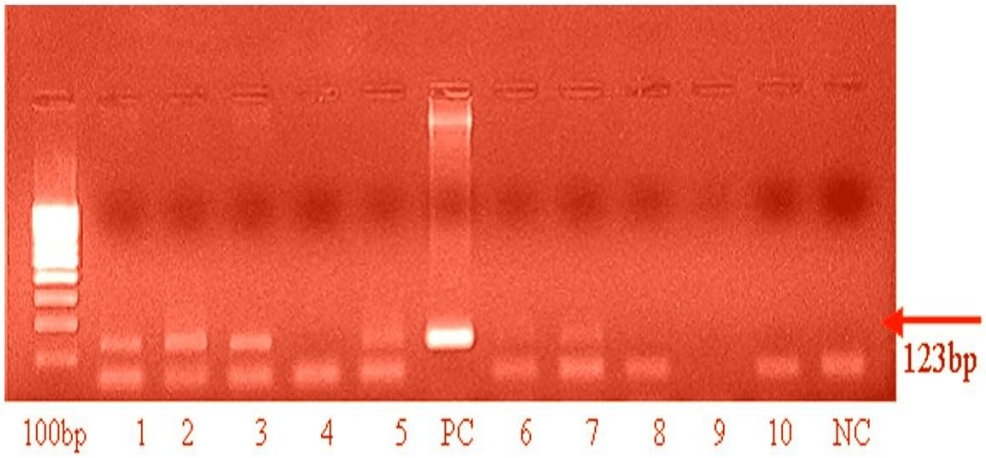


Plate XVIII

A 123 bp fragment of the M. tb amplified by PCR with primers IS 6110

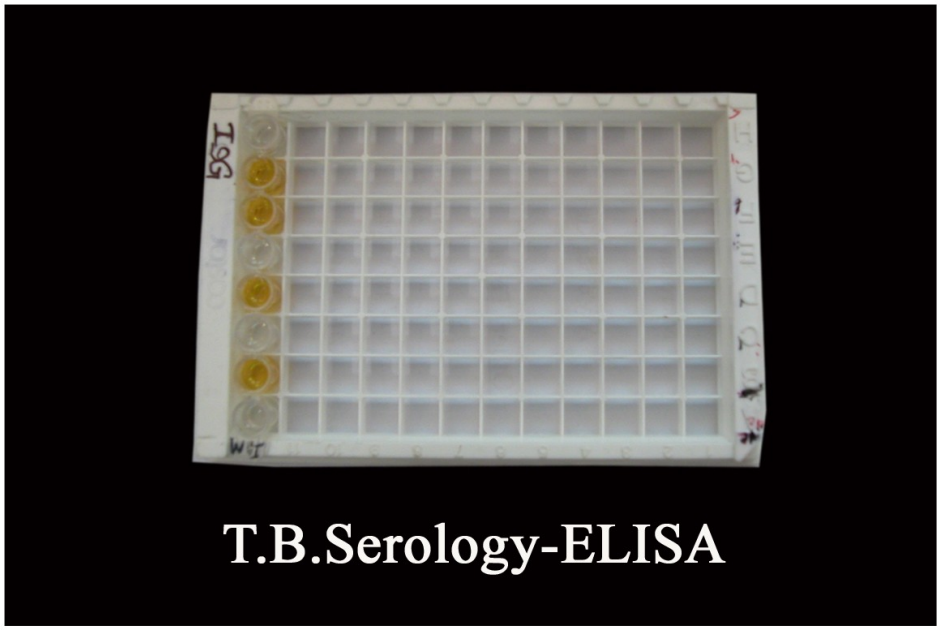


Plate XIX

ELISA test to detect IgG antibodies to A60 antigen complex

Plate XX

ELISA test to detect IgA antibodies to A60 antigen complex

5.4. RESULTS OF SEROLOGICAL METHODS

Table 10

A60 antibody class	Number of positive cases	Percentage of positivity
IgG + IgM – IgA -	12	11%
IgG – IgM + IgA -	16	15%
IgG – IgM – IgA +	6	6%
IgG + IgM + IgA -	18	17%
IgG + IgM – IgA +	8	8%
IgG – IgM + IgA +	8	8%
IgG + IgM + IgA +	22	21%
IgG – IgM – IgA -	15	14%

Among the 105 clinically proved cases, IgG alone positive in 60 (57%) cases (Plate XIX), IgM alone positive in 64 (61%) cases, IgA alone positive in 44 (42%) (Plate XX) cases whereas IgM and IgA were positive in 78 (75%) cases (Figure 8).

5.5 HIV /TB CO INFECTION

In our study we found out 3 cases were positive for HIV antibody by ELISA (Microlisa).

Discussion

DISCUSSION

In the present study all the 105 cases were diagnosed as TPE by clinical, radiological and Biochemical analysis of pleural fluid. The age and sex distribution in this study showed, among the 105 TPE cases, 10 were less than 20 years, and 95 were more than 20 years[92]. Among them 71 were male and 34 were female in the ratio of 2: 1. 30% of them were associated with textile industries and 46% of them were associated with other risk factors. All of them belongs to low socio economic status with poor education. This shows that Tuberculous pleural effusion was common among middle aged men, for whom predisposing factors are common.

The common presenting symptoms were chest pain (75%), cough (54%), fever (55%), dyspnoea (48%), constitutional symptoms (21%) and with multiple symptoms of chest pain, cough and fever (60%) , fever, loss of appetite and loss of weight (15%) and cough with haemoptysis (41%).

CATEGORIES OF PATIENTS:

Among the 105 clinically diagnosed TPE, 50 cases shows concomitant pulmonary findings. Three cases were proven as pulmonary tuberculosis and completed full course of ATT for one year under RNTCP. 25 cases were recently started on ATT on clinical grounds.

Categories of patients with concomitant pulmonary findings:

Category	Number
Apical P T with effusion	26
PT with effusion with mediastinal shift	2
Mid and lower lobe infiltration with effusion	5
Pyothorax	4
PT with hydropneumothorax	3
Bilateral PT with unilateral effusion	10
Pleural effusion	55

In our study 48% of patients with TPE have concomitant pulmonary findings. Sputum smear examination showed that 15 cases (14.3%) were smear positive. Among the 40 cases of unilateral PT with effusion only 5 (4.8%) were ZN positive whereas all the 10 cases of bilateral PT with effusion (9.5%) were smear positive. Sputum smear examination were negative in all other TPE cases where there is no pulmonary infiltration, even though earlier studies showed that even in the absence of underlying lung infiltrates smear was positive in 4 –11% cases of TPE[13,30,109]. So sputum smear examination is much helpful in the diagnosis of pleural effusion only when it is associated with pulmonary infiltrates.

Macroscopic examination of pleural fluid was cleal and straw coloured in most of the cases but serosanguinous fluid were also obtained

in few cases of TPE. Neutrophilic predominance were also seen in 10 – 15% of cases. These could be either due to active inflammation in the lung parenchyma with tuberculosis or in the early stages of tuberculosis.

In aspirates from tuberculous pleural effusion, organisms are rarely seen following ZN microscopy because of paucibacillary nature of the disease. In our study we have encountered only 4 (3.8%) ZN positive cases. Among the four, 3 were culture positive and one was culture negative. The culture negative case maybe due to the presence of non-viable mycobacteria in partially treated patients or due to harsh chemical treatment used in decontamination[19].

Mycobacterial culture, though considered as the "Gold Standard" was positive only in 20% of patients[13,43]. The low sensitivity of the culture in the pleural fluid was also demonstrated in a study done by Berger and Mejia et. al., who verified that in the pleural fluid cultures, it is only possible to detect the presence of the M. tuberculosis in 20 – 30% of infected patients[13]. In another study done by SK Katiyar and RP Singh et. al., culture was positive only in 19.05% of cases. In this study four cases of multi drug resistant tuberculosis were found. Another important consideration is the occurrence of natural drug resistant mutants.

The results are not surprising considering the fact that the pleural effusion in patients with tuberculosis mainly occurs as a result of delayed hypersensitivity reaction to Tuberculin proteins and the presence of bacilli in pleural fluid is not always necessary. Direct examination of pleural fluid and ZN staining requires bacillary concentration of more than 10,000 /ml and therefore has a low sensitivity. Although culture is more sensitive, it requires a minimum of 10 viable bacilli/ml.

Although, TPE may resolve over a period of several months with out treatment, failure to diagnose and treat pleural tuberculosis can result in progressive disease and the involvement of other organs in as many as 65% of patients. However, treatment based on clinical suspicion rather than on microbiological diagnosis results in over treatment, delay in accurate diagnosis and potentially greater morbidity. A remarkable delay is frequent in EPTB.

PCR is the rapid and sensitive method for the detection of Mycobacterial DNA in TPE (Nagesh et.al., 2001). The PCR protocol used in this study was based on the amplification of a portion of the mycobacterium tuberculosis genome located in the IS 6110 insertion sequence. Thierry and collaborators described that this sequence is

repeated approximately 20 times in the genome of mycobacteria and it is specific for the M. tuberculosis complex[132].

In our study all culture positive and smear positive cases were PCR positive. 2 NTM cases were also subjected to PCR but the PCR results were negative because the primers used were specific for M. Tuberculosis. Among the 85 culture negative cases PCR was positive in 53 cases. PCR was positive overall in 73 cases (70%). These data are in agreement with those obtained by Nagesh et. al., They reported a sensitivity of 70% and specificity of 100%.

Sensitivity and Specificity of PCR shown in various studies an listed in the Table below:

Reference	Sensitivity	Specificity
S. Nagesh, Jindal Et. Al., 2001	70%	100%
Portillo – Gomez L, Et.Al., 2000	94%	100%
Lazraq R, Guesdon JL Et. Al., 1999	93%	95%
Tan MF, Ng WC Et. Al., 1997	100%	88%
Gunisha P, Medhavan HN Et. Al., 2000	30%	95%
Almeda J, Gaenia A Et. Al., 2000	59%	97%

Negative PCR seen in 32 cases may be due either to inhibitors of Taq polymerase that are particularly high in pleural fluid or to sampling

difficulty or the causative agent may be NTM[19]. However, whenever the results are positive the Mycobacterial load must have been sufficiently high. Even though PCR is highly sensitive, expensive and laborious procedures cross contamination and false positive results should be taken into consideration.

Several serological methods have been proposed both in the past decades and more recently for the rapid diagnosis of TPE. They are characterized by simplicity, rapidity of technical execution as well financial viability and automation. The following reasons attracted the attention to establish the serological diagnosis.

1. Smear examination and in vitro culture of Mycobacteria yield low sensitivity as well as time consuming.

2. PCR claimed to be more sensitive but demands high level of skill, expensive set up and reagents.

3. All of the above methods demand sample collection from the site of infection which in many extra pulmonary situations may not be possible.

4. EPTB is difficult to diagnose as in many cases clinical & radiological findings are non-specific.

The first attempt of serodiagnosis reported by Arloing in 1898 since then several studies have been carried out by using quite complex antigens such as whole bacteria, culture filtrates, bacterial extracts and tuberculin's or PPD. The diagnostic value of these antigens have shown varying degree of success.

Enzyme linked Immunosorbant assay (ELISA) technique have been developed for the serodiagnosis of tuberculosis, which is a simple instrument with inexpensive reagents. Cocito Et. Al., in 1991 reported a novel antigen, A60, which could be successfully used to detect IgG, IgA and IgM class of antibodies using an ELISA technique.

The A60 antigen based kit was selected for evaluation because it has been used widely in our country as evident with the Indian Literature (Kalantri Y)[72]. Only few studies are available on the serodiagnosis of TPE. The purpose of this study is to evaluate the IgG, IgA and IgM antibody response to A60 antigen complex in TPE.

Sensitivity and Specificity of ELISA diagnostic kits:

(IgG antibody measurement)

Antigen	Sensitivity %	Specificity %

38 KDa	49 – 89	98 – 100
30 KDa	62 – 78	97 – 100
16 KDa	24 – 71	97 – 99
LAM	26 – 81	92 – 100
A60	71 – 100	71 – 95

(source : Serodiagnosis of Tuberculosis in children. S. Mahadevan, JIMA issue vol.100,No.2, Feb 2002)

The wide variation in the sensitivity of various antigens is due to the fact the most of the antigens are genus specific. There is wide spread sharing of epitopes between various species. Due to lack of a single species specific antigen various studies shows variable results.

In our study we have detected all the three antibodies using cut – off value given by the manufacture's protocol. We found out that IgG was positive in 57% of cases. This is a correlation with the studies of Charpin Et. Al., 1990 who found out a sensitivity of 48% and specificity of 71% for IgG. Caminero Et. Al., 1993 found out a sensitivity of 50 – 55% and specificity of 100% for IgG. Low sensitivity of IgG indicates that most of the patients are in the inactive stage of the disease.

IgM antibodies were positive in 61% of cases. This indicates that most of the patients are in active stage of the disease. In our study we have included 50% of the patients with pulmonary finding along with

effusion. High sensitivity of IgM indicates that effusion could be due to hypersensitivity reaction to tuberculin proteins. In 1990, Charpin Et. Al., showed sensitivity of 76% and specificity of 98% for IgM. In 2005, kalantri.Y. et.al., showed a sensitivity of 29 % and specificity of 96% for IgM.

IgA was positive in 44 (42%) cases. Gupta et.al., in 1995 showed a sensitivity of 92% and specificity of 90 % for IgG and IgA. In 1999 kaur. J et.al., showed a sensitivity of 64 to 70% and specificity of 80 to 82 % for IgG and IgA . IgM and IgA were positive in 78(75%) cases. In most of the studies, the cutoff value is standardized to a low level but in our study we have followed the manufacture's values. That could be one of the reasons for the low sensitivity of IgA. This should be further evaluated.

We found out that PCR was only 70 % sensitive where as IgM and IgA antibodies were 75%, sensitive so in peripheries where the PCR facilities are not available, Serodiagnosis (ELISA) can be carried out as a supplementary test in the diagnosis of Tuberculous pleural effusion. Since this is only a pilot study, serology needs further evaluation.

FOLLOW UP OF PATIENTS:

All the patients were started on ATT regime recommended under RNTCP. The follow up of these patients after 6 months showed drastic improvement in clinical symptoms and follow up X-ray chest (PA) also showed improvement. All the patients were satisfactory except one HIV-seropositive case, who died after 8 months of treatment. MDR –TB patients were given second line drugs.

Conclusion

CONCLUSION

- We have successfully established a panel of diagnostic tests for paucibacillary tuberculous plural effusion at Coimbatore Medical College, Coimbatore.

- Delay in diagnosis leads to sequelae as well as spread of infection to other organs.

- More than one diagnostic procedure is often needed for confirmation of TPE.

- Bacteriological, chemical (HPLC), molecular (PCR), and serological confirmation may aid in current diagnosis of TPE.

- Sputum smear examinations in TPE cases were useful only if there is concomitant pulmonary infiltrates.

- PCR is the rapid and sensitive test, which can be carried out where facilities are available.

- Drug susceptibility tests on culture positive cases will detect MDR – TB.

- Since NTM are resistant to all first line drugs, the principle of therapy for NTM requires attention.

- Despite of large number of studies carried out over the past several years, serology has found little place in the routine diagnosis of tuberculosis. Sensitivity and specificity depends on the antigen used.

- This study shows that serodiagnosis is as equally sensitive as PCR. So serology can be carried out as a diagnostic test along with clinical evidence to diagnose tuberculous pleural effusion cases especially in peripheral centers where PCR facilities are not available.

Summary

SUMMARY

- Total numbers of 105 cases of clinically diagnosed TPE were included in this study for demonstration of AFB both from sputum and pleural fluid and isolation of M. tuberculosis from pleural fluid.

- Among the 105 cases, sputum smear for AFB was positive in 15 cases, pleural fluid smear for AFB was positive in four cases. Culture was positive for M. tuberculosis in 19 cases and for NTM in two cases. All the culture positive cases were confirmed by HPLC.
- Four of the culture positive cases were found to harbor multi drug resistant tuberculosis. All the 105 pleural fluid samples were subjected to PCR which was positive in 73 cases.
- Sera collected from these patients were tested against A60 antigen complex.
- The study showed that IgM and IgA antibody response was good to A60 antigen complex. IgM and IgA were positive in 78 cases.
- Screening of the study subjects for the presence of HIV infection, detected 3 cases were dually infected with TB and HIV.

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Appendix

APPENDIX

1. Ziehl Neelsen Technique:

(a) Strong Carbol Fuchsin:

Basic Fuchsin	**:**	**10 gms.**
Absolute alcohol	**:**	**100 ml.**
Phenol	**:**	**50 gm.**
D. Water	**:**	**900 ml.**

Dissolve basic Fuchsin in absolute alcohol, add D. Water to it and then add phenol.

(b) Acid Alcohol

Conc. Hcl.	**:**	**20 ml**
Sodium Chloride	**:**	**20 gms.**
D. Water	**:**	**500 ml.**
Absolute Alcohol	**:**	**1500 ml.**

Dissolve sodium chloride in distilled water, add acid slowly by the side of the conical flask, dissolve it and then add alcohol to it.

(C) 1% Methylene Blue:

Methylene Blue	**:**	**1 µm**
D. Water	**:**	**100 ml.**

Dissolve methylene blue in distilled water.

2. Preparation of Middle Brook 7H9 Medium:

Salt Solution:

Ammonium sulphate	**:**	**0.50**

Disodium Phosphate	**:**	**2.50**
Monopotassium Phosphate	**:**	**1.00**
Sodium Citrate	**:**	**0.10**
Magnesium Sulphate	**:**	**0.25**
Calcium Chloride	**:**	**0.0025**
Zinc Sulphate	**:**	**0.001**
Copper Sulphate	**:**	**0.001**
Ferric Ammonium Citrate	**:**	**0.04**
L. Glutamic Acid	**:**	**0.50**
Pyridoxine	**:**	**0.01**
Protein	**:**	**0.0005**
Final pH	**:**	**6.6 ± 0.2.**

Suspend 2.35 gm in 450 ml of D. Water. Add 2 ml of glycerol and heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 Ibs pressure at 121˚c for 10 mts. Cool to 45˚c or below and aseptically add 1 vial of middle brook ADC growth supplement.

ADC growth Supplement:

Bovine Albumin, Fraction V:		**2.50 gm**
Dextrose	**:**	**1.00 gm**
Catalase	**:**	**0.0015 gm**
D. Water	**:**	**50 ml.**

Aseptically add 50 ml in 450 ml of sterile, middle brook 7H9 Broth Base. Mix well and pour into sterile screw capped tubes.

3. Preparation of LJ Medium:

Mineral Salt Solution:

Monopotassium Phosphate	**:**	**2.4 gms.**
Magnesium Sulphate	**:**	**0.24.**
Magnesium Citrate	**:**	**0.60.**
Potato Starch (soluble)	**:**	**30.00.**
L. Asparagine	**:**	**3.60-.**
Malachite Green	**:**	**0.40.**

Suspend 9.31gms in 150 ml of D. Water containing 3.ml glycerol. Boil to dissolve the medium completely. sterilize by autoclaving at 15 lbs pressure, 121˚c for 15 minutes.

Meanwhile prepare 250ml of whole egg emulsion collected aseptically. Add egg emulsion gently to base mix to obtain uniform mixture. Distribute in sterile screw capped bottles. Arrange bottles in a slanted position.

4. HPLC:

Materials:

1. **Saponification reagent** — **KOH**, **Methanol** → **Reagent grade water.**
2. **Acidification reagent** — **Conc. Hcl** → **Reagent grade water.**
3. **KHco3 reagent** — **KHco3**, **Methanol** → **Reagent grade water**
4. **Derivitization reagent.**
5. **Clarification reagent.**

Sample Preparation:

A. Cell harvesting procedure:

- **Add 0.2ml of saponification reagent**

- **1 - 2 loopful of growth on LJ medium.**
- **Vortex – 20 seconds.**

B. Saponification & extraction:

- **Autoclave 121˚c – 15 psi – 1 hour.**
- **Add 2.0ml of chloroform.**
- **1.5ml of acidification reagent. Cap tightly.**
- **Vortex 20 seconds.**

Allow the layers to separate 20 – 30 seconds. Using glass pasteur pipette, remove bottom layer (chloroform), containing mycolic acids and transfer to a new tube. Evaporate chloroform in water bath at 85 - 105ºc.

C. Derivitization to p. bromophenacyl esters:

- **Add 0.1ml of KOH.**
- **Evaporate at 85 - 105˚ c.**
- **Cool the sample and add 1.0ml of chloroform followed by 50µl of Derivitization reagent.**
- **Cap and vortex 30 sec.**
- **Heat at 85 - 105˚c for 20 minutes.**

D. Clarification by liquid – liquid extraction:

- **Cool and add 1.0ml of clarification reagent. Vortex 20 sec.**
- **Remove the bottom layer (chloroform) and transfer to a new tube.**

E. Completion and Storage:

Evaporate to dryness at 85 – 105˚c. Cap tightly store at 4 – 6 °c in the dark.

5. PCR

A. Experimental Procedure:

- **Transfer one to two loopful of specimen to an eppendorf tube**

Containing 500µl TE buffer (pH 8.0).

- **Heat 20mts at 80˚c to kill the organisms.**
- **Spin the sample in micro centrifuge for 2mts until a compact pellet forms. Discard the supernatant and resuspended pellet in 500µl TE buffer by repeated pipetting.**
- **Add 50µl of (10mg/ml) lysozyme, mix and incubate 2hr at 37˚c.**
- **Add 70µl of 10% SDS and 6µl of (10mg/ml) proteinase K.**
- **Mix and incubate 10min at 65˚c.**
- **Add 100µl of 5M Nacl and mix thoroughly.**
- **Add 80µl of CTAB – Nacl solution.**
- **Mix thoroughly and incubate for 10min at 65˚c.**
- **Add an approximately equal volume of chloroform/isoamyl alcohol mixture.**
- **Mix thoroughly, and spin in a micro centrifuge for 5min.**
- **Remove the aqueous supernatant to a fresh micro centrifuge tube.**
- **Add 600µl of isopropanol.**
- **Place at - 20˚c for 60minutes.**
- **Spin 15 min in a micro centrifuge at 10,000 rpm at 4˚c.**

B. Quantification of DNA:

When the DNA solution is of uniform viscosity, take 10µl and put it into a labeled 1.5ml tube and add 990µl sterile Milli Q water. Cap the tube, mix it and leave in fridge for 2 hours. Use the quartz cuvettes and TE buffer as the blank, measure the OD260nm and OD280nm of the samples. The ratio should be greater than 1.75.

A solution with an OD260 of 1 contains 30µg of DNA per milliliter. To calculate the concentration of the stock DNA, multiply by the dilution factor (i.e., 100 = 300µg/ml). To calculate the total amount of DNA, multiply by the total volume of the stock DNA (i.e., 0.4ml = 120µg).

C. PCR Protocols:

I. Reaction Volume

Typical 50µl reaction volume given below will be goodfor almost every PCR assay.

Components	Volume	Final conc. In 50µl
Milli Q Water	--------	------
10x Reaction buffer	5.0µl	1x
d NTP's Mix (10mM)	1.0µl	200µM
Primer # 1	1.0µl	20 pM/Reaction
Primer # 2	1.0µl	20 pM/Reaction
Taq DNA Polymerase (IU/µl)	1.0µl	1U/Reaction
Template	10.0µl	100ng

- **10x Reaction buffer: 10mM Tris – Hcl, 50mM kcl, 1.5mM $Mgcl_2$ Triton X 100**
- **d NTP's Mix : A mixture of d ATP, d GTP, d CTP and d TTP**
- **Primer # 1 : TB 4**
- **Primer # 2 : TB 5**

Reconstitute the primer with TE buffer as per requirements.

- **Taq DNA Polymerase: Ready to use.**

II. Pipetting and DNA Template

Pipetting can be done under a laminar flow of sterile air and using aerosol – resistant pipette tip.

III. Thermocyclers and PCR vials.

While using older model thermocyclers (without a heated lid) mineral oil is necessary to cover the reaction mixture.

D. Detection and analysis of PCR Product:

- **Prepare 2% agarose gel using 1x electrophoresis buffer by melting it in a microwave oven.**
- **Add (1µl of stock/50ml) ethidium bromide to the gel when the gel reaches 50˚c . Pour the mixture into the sealed gel casting platform.**
- **After gel has hardened remove the seal from the gel – casting platform and remove the gel comb. Place the platform into electrophoresis tank containing sufficient electrophoresis buffer to cover the gel.**
- **Prepare DNA sample with loading buffer (18µl of PCR product + 2µl gel loading buffer) and load samples, marker DNA into the wells with a pipettor.**
- **Attach the lids and set the power pack.**
- **Turn off the power supply when the bromophenol blue dye from the loading buffer has migrated a distance judged sufficient for separation of DNA fragments.**
- **Observe for the PCR product under Transilluminator.**

6. ELISA: (Anda TB)

Kit Components:

1. **Sample diluent (light green).**
2. **Peroxidase – labeled anti human immunoglobulin in stabilized buffer (dark blue).**
3. **10 – fold concentrated TMB (Tetramethyl benzidine).**
4. **TMB diluent citric acid – phosphate buffer containing 0.02% of H_2O_2.**
5. **Sulpheuric acid 0.5N (Stop solution - red).**
6. **IgG and IgA references: Negative (yellow), 1U/ml (white), 2U/ml (Purple), 4U/ml (Pink), 8U/ml (Bright Red) and 16U/ml (Light blue).**
7. **IgM references: Negative (grey) and Positive (brown) limit.**

8. **20. Fold concentrated wash buffer solution.**

9. **Break apart strips of wells coated with antigen A60 from M. bovis (BCG).**

Reagent Preparation:

A. Washing solution:

Add 19 volumes distilled water to one volume of concentrated (20x) washing buffer. In general 1litre is needed to wash one plate: add 950 ml D. water to 50 ml concentrated (20x) washing buffer.

B. TMB Work Solution:

Add 9 volumes of TMB diluent to one volume of conc. (10X) TMB solution.

7. ELISA (MICROLISA):

Kit Components:

1. **Break way micro wells coated with HIV – 1 & HIV – 2 recombinant proteins packed in a pouch with dessicant.**

2. **Sample diluent.**

3. **Enzyme conjugate concentrate (100x) – Anti human IgG labeled with horse radish peroxidase with protein stabilizers.**

4. **Conjugate diluent (Buffer Containing Stabilizers).**

5. **Wash buffer Concentate (25x) PBS with surfactant. Dilute 1 : 25 with distilled water before use.**

6. **TMB concentrate (100x) – to be diluted before use.**

7. **Substrate (TMB diluent) – Buffer containing subtatie.**

8. **Negative Control : Ready to use, normal human serum negative for HIV – 1, HIV – 2, HCV and HBs Ag and HCv,**

9. **Positive Control : Ready to use, inactivated and diluted human serum non reactive for HBs Ag and HCV, positive for HIV antibodies.**

10. Stop Solution : Ready to use 2N sulpharic acid,.

Preparation of reagents:

A. Sample Preparation: Micro well dilution.

- **Pipette 100µl of sample diluent into the micro well.**
- **Add 10µl of serum sample of ensure thorough mixing.**

B. Preparation of wash buffer:

Prepare at least 50ml (2ml concentrated buffer with 48ml water) of buffer for each Microlisa strip used. Mix well before use.

C. Preparation of working conjugate:

Dilute conjugate concentrate 1: 100 in conjugate diluent. Prepare a fresh dilution for each assay and mix thoroughly before use.

D. Preparation of Working substrate solution:

Dilute TMB concentrate 1: 100 in substrate, TMB diluent.